Remarks

Election of Species Requirement

Claims 1, 3-5, 16 and 18 are pending. The examiner requested that applicant cancel all non-elected embodiments from the claims. Applicants respectfully remind the examiner of U.S. 37 CFR 1.146, which states that in the first action on an application containing a generic claim to a generic invention (genus) and claims to more than one patentably distinct species embraced thereby, the examiner may require the applicant in the reply to that action to elect a species of his or her invention to which his or her claim will be restricted if no claim to the genus is found to be allowable. The requirement for restriction is for search purposes. No art having been found which discloses the elected species SEQ ID No. 15, the examination of the remaining species should now be conducted.

Rejection Under 35 U.S.C. § 112, first paragraph (new matter)

Claims 1, 3-5, 16 and 18 were rejected under 35 U.S.C. § 112, first paragraph, as containing new matter. Applicant respectfully traverses this rejection.

The previously filed amendment cited to specific points in the specification where support for the amendments to claim1 was found:

Claim 1 has been amended to recite that the method is for inhibiting binding of a cell to collagen, glycosaminoglycan, fibrinogen, fibronectin, collagen, vitronectin, thrombospondin, osteopontin, bone sialoprotein 1, von Willebrand's factor or vascular adhesion molecule and includes providing the cell with a peptide molecule comprising a peptide having a molecular weight between 100 and 2500 Daltons and consisting of a sequence selected from the group consisting of SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12,

SEQ ID NO:14 and SEQ ID NO:15. Support for this amendment is found on page 3, line 15 and lines 23-29; page 8, lines 17-21; page 19, lines 21-22; and page 20, lines 5-8.

The specification on page 2, lines 26-32 describe the present invention as pertaining to adhesion modulatory peptides designed to promote an/or enhance the adhesion of specific cells or cell types based on adhesion receptors expressed by the specific cell or cell type, one aspect being a method of modulating adhesion of a target cell to a substrate. The term substrate is defined to include physical materials as well as molecular components (extracellular matrix components, collagen and glycosaminoglycans (page 3, lines 2-6). The specification at least on page 4, lines 25-29 further describes the adhesion modulatory peptide as being able to inhibit binding of an adhesion receptor predominantly expressed expressed by the target cell. Finally, the specification at least from page 5 line18 till page 8 sets forth the ligands to the adhesion receptors. Therefore, "glycosaminoglycan, fibrinogen, collagen, vitronectin, thrombospondin, osteopontin, bone sialoprotein 1 and von Willebrand's factor" in claim 1 do not constitute new matter.

The examiner's position that the exact phrase is not associated with SEQ ID No. 15 is without merit. Claim 1 is not limited to SEQ ID NO:15 but to a class of peptides, of which SEQ ID NO:15 is one.

Rejection Under 35 U.S.C. § 112, first paragraph (enablement)

Claims 1, 3-5, 16 and 18 were rejected under 35 U.S.C. § 112, first paragraph, as not being enabled. Applicant respectfully traverses this rejection.

The Legal Standard

Any analysis of whether a particular claim is supported by the disclosure in an application requires a determination of whether that disclosure, when filed, contained sufficient

information regarding the subject matter of the claims as to enable one skilled in the pertinent art to make and use the claimed invention, *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404 (Fed. Cir. 1988). See also *United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988) ("The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation."). The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. The Supreme Court also noted that all of the factors need not be reviewed when determining whether a disclosure is enabling *In re Amgen, Inc. v. Chugai Pharm. Co.*, 927 F.2d 1200, 1213, 18 USPQ2d 1016, 1027 (Fed. Cir. 1991) (noting that the *Wands* factors 'are illustrative, not mandatory. What is relevant depends on the facts.'). As long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement of 35 U.S.C. 112 is satisfied. *In re Douglas v. United States* 510 F.2d 364; 184 U.S.P.Q. 613 (Ct. Cl.1975) the Court of Claims noted that a patentee cannot "be expected to foresee every technological problem that may be encountered in adapting his idea to a particular use. Some experimentation and exercise of judgment is to be expected. *In re Mineral Separation v. Hyde* 242 U.S. (1916), the court emphasized that some inventions cannot be practiced without adjustments being made to adapt them to the particular context. In such a situation, a specification is sufficient if it gives adequate guidance to one skilled in the art on how such adjustments are to be made

Analysis

The specification on page 1 lines 15 to 33 discusses the proteins involved in cell adhesion, which are well known in the art. The specification on page 14-17 discloses how to

make the adhesion modulatory peptides and on page10-11 (Table II), a method of how to use the claimed peptides to modulate cell adhesion. The specification on page 10 (Table II) discloses that SEQ ID NO: 15 can be used to inhibit the interaction between the integrinVLA-4 and its ligand VCAM interaction.

Additionally it is known in the art that VLA-4 has other ligands other than fibronectin and VCAM , [see, for example, Yabkowitz, *et al.* J. of Immunol, 151:149-158 (1993), Isobe, *et al.*, JBC, 272:8447-8453, (1997) and Bayless, et al. J. Cell Sci., 111:1165-1174 (1998) (attached)]. The specification need not disclose what is well-known to those skilled in the art and preferably omits that which is well-known to those skilled and is already available to the public. Furthermore, the specification on page 9, lines 1-9 sets forth the relative ease with which one skilled in the art would determine an adhesion receptor pattern for a particular cell type. The specification need not contain an example if the invention is otherwise disclosed in such manner that one skilled in the art will be able to practice it without an undue amount of experimentation.

The examiner has provided only speculation and unsupported arguments for why the specification is not enabling. It is well established that a specification is presumed to be enabling. A *prima facie* case of non-enablement can only be made upon a showing of evidence ***(not argument)*** of why one skilled in the art would not be able to make and use the claimed subject matter. Even assuming *arguendo* that the examiner has done so, applicant has rebutted this with reference to support not only in the specification but in the literature.

The specification is therefore enabling for the use of the peptide molecules of claim 1. It is clear that one of ordinary skill in the art would be able to inhibit the binding of a cell outside of a subject to the recited substrates by providing the cell with a peptide molecules recited in claim 1.

Rejection Under 35 U.S.C. § 112, first paragraph (written description)

Claims 1, 3-5, 16 and 18 were rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor had possession of the claimed invention. Applicants respectfully traverse this rejection.

The Legal Standard

An applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention see *In re Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997). Possession may be shown in a variety of ways including description of an actual reduction to practice, or by showing that the invention was "ready for patenting" such as by the disclosure of drawings or structural chemical formulas that show that the invention was complete, or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention. See, e.g., *Pfaff v. Wells Elecs., Inc.*, 525 U.S. 55, 68, 119 S.Ct. 304, 312, 48 USPQ2d 1641, 1647 (1998); *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406; *Amgen, Inc. v. Chugai Pharmaceutical*, 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991) (one must define a compound by "whatever characteristics sufficiently distinguish it).

A "representative number of species" means that the species which are adequately described are representative of the entire genus. Thus when there is substantial variation within the genus, one must describe a sufficient variety of species to reflect the variation within the

genus. On the other hand, there may be a situation where one species adequately supports a genus. See, e.g., *Rasmussen*, 650 F.2d at 1214, 211 USPQ at 326-27.

Analysis

From the description in the specification, it is clear that the Applicants were in possession of the claimed subject matter at the time of filing. The specification on pages 14-17 describe how one can make the peptides to be used in inhibiting cell binding, the specification at least on pages 9-10 (Table II) discloses the amino acid composition of the peptides recited in claim 1 and a distinguishing characteristic for each. The structural description of integrins, glycosaminoglycan or vascular adhesion molecules are well known in the art. As affirmed by the Court in *Spectra-Physics, Inc. v. Coherent, Inc.*, 827 F.2d 1524 (Fed. Cir. 1987), a patent need not teach, and preferably omits, what is well known in the art.

The examiner is correct that claim 1 is not limited to *in vitro* binding. However, it clearly encompasses *in vitro* binding, which has been demonstrated as described in the application. Therefore applicants were clearly in possession and described that possession in the application as filed and claims 1, 3-5, 16 and 18 satisfy the written description requirement.

Allowance of claims 1, 3-5, 16 and 18 is respectfully solicited.

Respectfully submitted,

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Dated: December 22, 2005

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Journal of Cell Science 111, 1165-1174 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JCS1517

Osteopontin is a ligand for the $\alpha_4\beta_1$ integrin

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Accepted 12 February; published on WWW 20 April 1998

SUMMARY

Recent work has shown that osteopontin expression is upregulated at sites of cardiovascular injury. It has been hypothesized that osteopontin provides an adhesive matrix for endothelial and smooth muscle cells during remodeling of the vascular wall following injury. Osteopontin has also been found to be synthesized by monocytes and macrophages within injury sites. Here, we present data showing that osteopontin can promote leukocyte adhesion through the $\alpha_4\beta_1$ integrin. In the presence of physiologic concentrations of Mg^{2+} and Ca^{2+}, osteopontin purified from bovine milk promoted cell-substrate adhesion of HL-60 and Ramos cells, two model leukocyte cell lines. As with other adhesive ligands, adhesion to osteopontin required leukocyte activation. Under these conditions, no adhesion to control substrates such as bovine serum albumin was observed. Leukocyte adhesion was inhibited by anti-integrin antibodies directed at either the α_4 or β_1 integrin subunits but not by control antibodies directed to other integrins. Further adhesion experiments revealed that leukocyte binding to osteopontin was completely inhibited by an $\alpha_4\beta_1$-binding peptide containing the leucine-aspartate-valine (LDV) sequence, while a control, non-binding peptide containing leucine-glutamate-valine (LEV) had minimal effects. Affinity chromatography using either surface labeled HL-60 or Ramos cell extracts revealed that the $\alpha_4\beta_1$ integrin specifically bound to osteopontin. Immunoprecipitation of eluted fractions from these columns positively identified the $\alpha_4\beta_1$ integrin. In order to localize potential $\alpha_4\beta_1$-binding sites within osteopontin, the protein was proteolytically cleaved with thrombin. A 30 kDa N-terminal osteopontin fragment purified using fast protein liquid chromatography promoted $\alpha_4\beta_1$ dependent leukocyte adhesion in a manner similar to that of the intact protein. These data collectively demonstrate that the $\alpha_4\beta_1$ integrin is a new adhesion receptor for osteopontin and that an $\alpha_4\beta_1$ binding site exists in the NH_2-terminal thrombin fragment of osteopontin.

Key words: Osteopontin, $\alpha_4\beta_1$, Integrin, Leukocyte, Adhesion, Injury

INTRODUCTION

Osteopontin (OPN) is an acidic, phosphorylated matrix protein that contains an Arg-Gly-Asp (RGD) cell attachment sequence (Oldberg et al., 1986) and has been identified as an adhesive and migratory substrate for several cell types. OPN has previously been shown in vitro to promote adhesion and migration of vascular smooth muscle (Liaw et al., 1994, 1995b; Bayless et al., 1997) and endothelial cells (Liaw et al., 1994, 1995a,b; Bayless et al., 1997). Peritoneal or subcutaneous injection of OPN results in mononuclear cell and/or neutrophil (PMN) accumulation (Singh et al., 1990), suggesting that OPN may provide migratory and adhesive signals for leukocytes. OPN expression is observed at the intimal edge of a balloon-injured artery (Giachelli et al., 1991, 1993; Liaw et al., 1995a) and its expression correlates with the presence of macrophages in several types of renal injury (Giachelli et al., 1994; Pichler et al., 1994). This evidence indicates that OPN may participate in smooth muscle cell, endothelial cell and leukocyte adhesion and/or migration within injury sites.

Most cells adhere to OPN through integrins. The $\alpha_v\beta_1$ (Liaw et al., 1995b; Hu et al., 1995a). $\alpha_v\beta_3$ (Miyauchi et al., 1991; Ross et al., 1993; Liaw et al., 1994, 1995b; Hu et al., 1995b) and $\alpha_v\beta_5$ (Liaw et al., 1995b; Hu et al., 1995a) integrins bind OPN in an RGD-dependent manner. Recent evidence suggests that OPN is capable of promoting cell adhesion through sites other than its RGD site (Van Dijk et al., 1993; Nasu et al., 1995; Katagiri et al., 1996; Smith et al., 1996). Other integrins such as $\alpha_4\beta_1$ (Nasu et al., 1995), $\alpha_5\beta_1$ (Nasu et al., 1995) and $\alpha_9\beta_1$ (Smith et al., 1996) have been suggested as potential OPN receptors that may bind these alternative sites. However, further work is necessary to investigate the role of these other integrins in OPN-mediated cellular events.

The $\alpha_4\beta_1$ integrin is expressed on leukocytes (Lobb and Hemler, 1994; Springer, 1994; Carlos and Harlan, 1994), differentiated vascular smooth muscle cells (Duplaa' et al., 1997) and tumor cells (Taichman et al., 1991; Qian et al., 1994). The $\alpha_4\beta_1$ integrin has been shown to mediate cell-cell attachment as well as cell-substrate adhesion to extracellular matrix and other proteins (Hynes, 1992; Lobb and Hemler, 1994; Springer, 1994; Carlos and Harlan, 1994; Kilger and Holzmann, 1995; Davis et al., 1997). This integrin appears to play a critical role in the control of the inflammatory response in disease states such as diabetes, encephalomyelitis (Yednock

et al., 1992; Yang et al., 1993; Lobb and Hemler, 1994) and graft rejection (Molossi et al., 1995) in that administration of anti-$\alpha_4\beta_1$ blocking reagents inhibited the development of the diseases. The $\alpha_4\beta_1$ integrin has been reported to bind the CS-1 and CS-5 alternatively spliced domains of fibronectin (Humphries et al., 1987; Guan and Hynes, 1990; Mould et al., 1991) as well as VCAM-1, an inducible leukocyte-endothelial cell adhesion molecule (Elices et al., 1990). Known binding sequences for the $\alpha_4\beta_1$ integrin are Leu-Asp-Val (LDV) in the CS-1 region of fibronectin (Guan and Hynes, 1990; Komoriya et al., 1991; Wayner and Kovach, 1992) and Glu-Ile-Asp-Ser-Pro-Leu (QIDSPL) in VCAM-1 (Osborn et al., 1994; Vonderheide et al., 1994; Clements et al., 1994; Jones et al., 1995). Other identified recognition sites for $\alpha_4\beta_1$ within fibronectin include Ile-Asp-Ala-Pro-Ser (IDAPS) (Mould and Humphries, 1991), Arg-Glu-Asp-Val (REDV) (Mould et al., 1991) and Arg-Gly-Asp (RGD) (Sanchez-Aparicio et al., 1994).

In this study, we show that leukocytes can adhere to OPN through the $\alpha_4\beta_1$ integrin. We have shown this interaction by utilizing anti-integrin blocking antibodies directed to the α_4 or β_1 integrin subunits and $\alpha_4\beta_1$-specific binding peptides which blocked leukocyte binding to OPN. Affinity chromatography experiments demonstrated direct binding of $\alpha_4\beta_1$ to OPN. Leukocyte adhesion to an NH_2-terminal thrombin fragment of OPN was also mediated through $\alpha_4\beta_1$. Collectively, these data show that the $\alpha_4\beta_1$ integrin can mediate cellular adhesive events through OPN.

MATERIALS AND METHODS

Cell adhesion assays

Cell adhesion assays were performed to determine the ability of isolated OPN to promote leukocyte adhesion. Polystyrene microwells (Corning-Costar, Cambridge, MA) were coated with 50 µl of bovine OPN purified as previously described (Bayless et al., 1997) at a concentration of 20 µg/ml in Tris-buffered saline (TBS) overnight at 4°C. OPN was purified from bovine milk using sequential ion-exchange and hydrophobic chromatographic steps yielding ~8 mg of OPN per liter of milk. Purity (>95%) was assessed using Sypro-Orange staining of SDS-PAGE gels (showing a single 60 kDa band) and a single NH_2-terminal sequence as previously described (Bayless et al., 1997). After blocking with 10 mg/ml BSA (bovine serum albumin) (Sigma, St. Louis, MO) in TBS, wells were rinsed with Puck's Saline A (PSA) (Gibco-BRL, Grand Island, NY). HL-60 promyelocytic leukemia cells or Ramos lymphoblastoid cells (ATCC, Rockville, MD) were grown in RPMI-1640 (Gibco-BRL) and 10% fetal calf serum. Cells were rinsed and resuspended in PSA at a density of 100,000 cells/well. Medium for adhesion in all HL-60 cell experiments contained a final concentration of 100 µg/ml BSA in PSA. Concentrations of $CaCl_2$ and $MgCl_2$ were varied as described in the results. HL-60 cells were activated with the β_1-activating antibody, 8A2 (Kovach et al., 1992) at a concentration of 1 µg/ml and a phorbol ester, 12-0-tetradecanoyl phorbol 13-acetate (TPA) at a concentration of 50 ng/ml. Ramos cells were activated with the 8A2 monoclonal antibody alone at 1 µg/ml. After plating, cells were allowed to adhere for one hour at which time they were fixed with formalin. Plates were stained with 0.1% Amido Black for 30 minutes, rinsed and solubilized with 2 N NaOH to obtain an absorbance reading at 595 nm which corresponds directly to the number of cells stained in each well (Davis and Camarillo, 1993).

To determine which integrins were expressed on the cell surface of Ramos and HL-60 cells, cell binding experiments were performed using wells coated with anti-integrin antibodies. The cells' ability to attach to an antibody is governed by whether or not the cells express a given antigen. Antibodies tested include HP2/1 (α_4), Mab13 (β_1), Fib 504 ($\alpha_4\beta_7$), Mab16 (α_5) and TS1/22 (α_L). Control (blank) wells were coated with 10 mg/ml BSA. Wells were blocked with BSA and rinsed. Cells were added at 100,000 cells per well in the presence of 100 µg/ml BSA and the absence of cations. These experiments were performed with HL-60, Ramos and RPMI 8866 cells. The RPMI 8866 cell line is known to express $\alpha_4\beta_7$ (Erle et al., 1994) and was kindly provided by Drs David Erle and Russell Pachynski (University of California, San Francisco). Cells were allowed to adhere for 30 minutes and quantitated as described above. Fluorescence-activated cell sorting (FACS) was also performed with the same mouse anti-human antibodies used in the antibody binding experiments. Approximately 10^6 leukocytes per group were labeled with 5 µg of primary antibody for 30 minutes on ice and rinsed twice with 500 µl PBS. Goat anti-mouse secondary antibody conjugated to FITC (Dako, Glostrup, Denmark) was then added (10 µl) for 30 minutes and rinsed twice with 500 µl of PBS. FACS analysis was conducted using FACSCalibur (Becton-Dickinson) at the Center for Flow Cytometry and Image Analysis of the Institute for Molecular Pathogenesis and Therapeutics, Texas A&M University.

To determine which integrins were responsible for leukocyte adhesion to OPN, HL-60 and Ramos cells were incubated with various concentrations of divalent cations in combination with anti-integrin monoclonal antibodies or synthetic peptides specific for various integrins. Following a 15 minute preincubation period at 37°C with either antibodies or peptides, cells were activated with 1 µg/ml 8A2 and (for HL-60 cells) 50 ng/ml TPA, plated and allowed to adhere for one hour before fixing. In the antibody blocking experiments, HL-60 cells were incubated with the following monoclonal antibodies directed to integrin subunits at a concentration of 20 µg/ml: α_4 (HP2/1; supernatant used at 1:10 dilution; Francisco Sanchez-Madrid, Spain) (Sanchez-Madrid et al., 1986), α_4 (Immunotech, Westbrook, ME), β_1 (Mab_{13}; Becton Dickinson, Bedford, MA) (Akiyama et al., 1989), $\alpha_4\beta_7$ (Fib 504; Pharmingen, San Diego, CA) (Andrew et al., 1994), α_L (TS1/22; ATCC, purified as in the method of Larson, 1989) and α_5 (Mab_{16}; Becton Dickinson) (Akiyama et al., 1989). To further investigate the involvement of the $\alpha_4\beta_1$ integrin in adhesion to OPN, the Glu-Ile-Leu-Asp-Val-Ser-Pro (EILDVSP) synthetic peptide (Peninsula Labs, Belmont, CA) (Komoriya et al., 1991) and its control Glu-Ile-Leu-Glu-Val-Ser-Pro (EILEVSP) were pre-incubated with cells at a concentration of 250 µg/ml. Other peptides tested included the Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP, Gibco BRL) and its control, Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) (Pierschbacher and Ruoslahti, 1984). These peptides were also added at 250 µg/ml. Following the incubation period, cells were seeded and the assay performed as described above.

Isolation of integrins using osteopontin-Sepharose

To illustrate the integrin-binding capacity of OPN, purified OPN was coupled to cyanogen-bromide 4B (Sigma) at 2 mg/ml according to the manufacturer's instructions. HL-60 and Ramos cells were surface biotinylated as described (Davis, 1992). A 50 µl pellet of cells was extracted with 1 ml 3% octylglucoside (ICN, Irvine, CA) in TBS containing 1.5 mM Mg^{2+}, 1.5 mM Mn^{2+} and 10^{-3} M phenylmethanesulfonic acid. The cell extracts were mixed at 5-10 minute intervals with OPN-Sepharose (0.9 ml) over a 2 hour period at 0°C. The column was washed with 20 ml of 1% octylglucoside plus cations and 0.5 ml fractions were eluted with 3 ml of 1% octylglucoside + 10 mM EDTA. 30 µl of each fraction were loaded and run under non-reducing conditions on a 7% acrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). The membranes were blocked overnight at 4°C with 5% non-fat dry milk in water and developed for alkaline phosphatase activity as previously described (Bayless et al., 1997).

Integrin immunoprecipitation

Integrins which bound to the OPN-Sepharose column were identified using immunoprecipitation. Sepharose beads conjugated with goat anti-mouse IgG (Sigma) or with Protein A (Sigma), were rinsed and suspended 1:1 with 0.5% Triton X-100 in TBS. In 1.5 ml microcentrifuge tubes, 200 µl of the bead mixture was added to 5 µg of monoclonal antibodies against several human integrin subunits including α_4 (HP2/1, Immunotech), α_4 (P4C2, Gibco-BRL), β_1 (LM534), α_5 (MAB_{16}) and α_L (TS1/22) or 10 µl of a rabbit polyclonal antibody directed to the α_4 subunit cytoplasmic domain (Chemicon, Temecula, CA). These mixtures were then combined with 300 µl of pooled EDTA eluate from OPN-Sepharose and 700 µl of 0.5% Triton X-100 in TBS. This mixture was rotated continuously at 4°C overnight after which time tubes were centrifuged and rinsed six times with 1 ml of 0.5% Triton X-100 in TBS. 75 µl of 2× sample buffer was added to the beads and this mixture was boiled for 5 minutes. 30 µl samples were run on 7% SDS-PAGE under non-reducing conditions and transferred to PVDF. The blots were probed for biotin using streptavidin-alkaline phosphatase as described above.

Thrombin cleavage of OPN

Bovine thrombin (American Diagnostica, Greenwich, CT) was coupled to cyanogen-bromide 4B at 200 µg/ml according to the manufacturer's instructions. OPN was incubated with the beads for 2 hours at 37°C and gently mixed every 10 minutes. Following cleavage, the beads were pelleted and supernatant containing thrombin-cleaved OPN was aliquoted and frozen at –20°C.

Purification of an N-terminal thrombin fragment of OPN using fast protein liquid chromatography (FPLC)

The fragments of OPN produced after thrombin treatment were isolated and purified using FPLC. Approximately 100 µg of thrombin-cleaved OPN in PBS was dissolved in 6 M urea and 10 mM Tris (200 µl total) and applied to a Q-Sepharose column (Pharmacia-Biotech; Alameda, CA) at a flow rate of 0.8 ml per minute. The column was washed with 5 ml of buffer A (0 M NaCl, 10 mM Tris, 6 M urea). Following the wash, bound proteins were eluted using a 0-1.0 M NaCl gradient in 6 M urea and 10 mM Tris-HCl over 20 minutes. Fractions (600 µl) were collected and analyzed using SDS-PAGE and visualized with a copper staining method (Bio-Rad). The N-terminal OPN fragment eluted consistently at 0.5 M NaCl. The purified fragment was subjected to amino-terminal sequencing using the Edman method on a Hewlett Packard G1005A protein sequencing system at the Biotechnology Instrumentation Facility in the Department of Entomology at Texas A&M University.

RESULTS

Leukocyte adhesion to OPN occurs in the presence of physiologic levels of divalent cations

A model leukocyte cell line, HL-60, was used to investigate whether OPN promoted leukocyte adhesion. Fig. 1 shows the adhesion of HL-60 cells to OPN in the presence of various concentrations of Ca^{2+} and Mg^{2+} and in the presence or absence of leukocyte activation using TPA and 8A2. Cell attachment to OPN was greatest with 3 mM Mg^{2+} alone and decreased with increasing Ca^{2+}/Mg^{2+} ratios. However, substantial adhesion to OPN substrates occurred with physiologic concentrations of divalent cations (2 mM Ca^{2+}, 1 mM Mg^{2+}). Adhesion to the control substrate, BSA was insignificant in all groups, regardless of activation. A second leukocyte cell line, Ramos, also attached to OPN in the presence of 2 mM Ca^{2+}, 1 mM Mg^{2+} (see later on) but not in

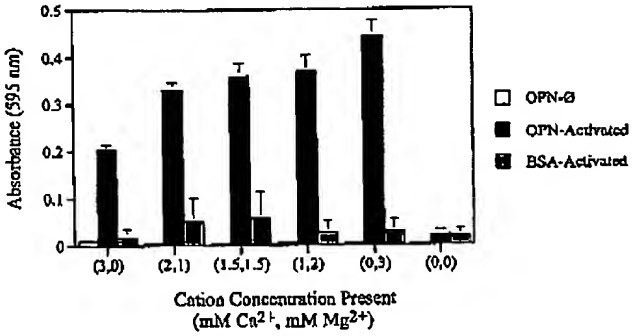

Fig. 1. Leukocyte adhesion to OPN requires activation and the presence of divalent cations. HL-60 cells were activated with the β1-activating antibody 8A2 (1 µg/ml) and with a phorbol ester, TPA at 50 ng/ml. The divalent cations Mg^{2+} and Ca^{2+} were then added at the concentrations indicated and allowed to adhere to wells coated with either OPN (20 µg/ml) or bovine serum albumin (BSA) (10 mg/ml). Experiments were performed and quantitated as described in Materials and Methods. The data shown are from a representative experiment (n=3) performed in triplicate wells and values shown are mean absorbance ± s.e.m.

the absence of divalent cations (not shown). Ramos cell attachment required activation with only the 8A2 antibody. No adhesion of either leukocyte cell line occurred in the absence of divalent cations, supporting the concept that this adhesion was mediated through integrins. Also, the activating influence of 8A2 (Kovach et al., 1992) strongly suggested the involvement of a β_1 integrin in leukocyte adhesion to OPN.

Integrin expression by HL-60 and Ramos cell lines

To assess which integrins were responsible for leukocyte adhesion to OPN, adhesion experiments to substrate bound anti-integrin antibodies and fluorescence-activated cell sorting (FACS) were performed. As shown in Fig. 2A, HL-60 and Ramos cells adhered to microwells coated with anti-integrin antibodies directed to α_4 and β_1 but not $\alpha_4\beta_7$. In contrast, a control cell line which is known to express $\alpha_4\beta_7$, RPMI 8866, bound to anti-α_4 and anti-$\alpha_4\beta_7$ coated microwells but not anti-β_1 wells. Also shown are the binding of these cells to anti-α_L and -α_5 integrin antibodies. A FACS experiment in Fig. 2B confirms the cell adhesion data. HL-60 cells express $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_L\beta_2$ while Ramos cells express $\alpha_4\beta_1$. The only integrin commonly expressed between the two cell lines is $\alpha_4\beta_1$. Neither cell line expresses $\alpha_4\beta_7$ or $\alpha_v\beta_3$ (Fig. 2).

The $\alpha_4\beta_1$ integrin mediates leukocyte adhesion to OPN

Cell adhesion experiments with integrin blocking reagents were performed to identify the integrins responsible for the adhesion of HL-60 and Ramos cells to OPN. Cells were incubated with antibodies directed to HL-60 or Ramos cell integrin subunits and plated on OPN. The results are shown in Figs 3A (HL-60) and 4A (Ramos). The addition of either anti-α_4 or anti-β_1 antibodies markedly reduced leukocyte adhesion to OPN compared to control. Antibodies directed toward $\alpha_4\beta_7$, α_5 and α_L had no effect. To further confirm that the $\alpha_4\beta_1$ integrin is involved in leukocyte adhesion to OPN, integrin-binding peptides were utilized (Figs 3B and 4B). The LDV

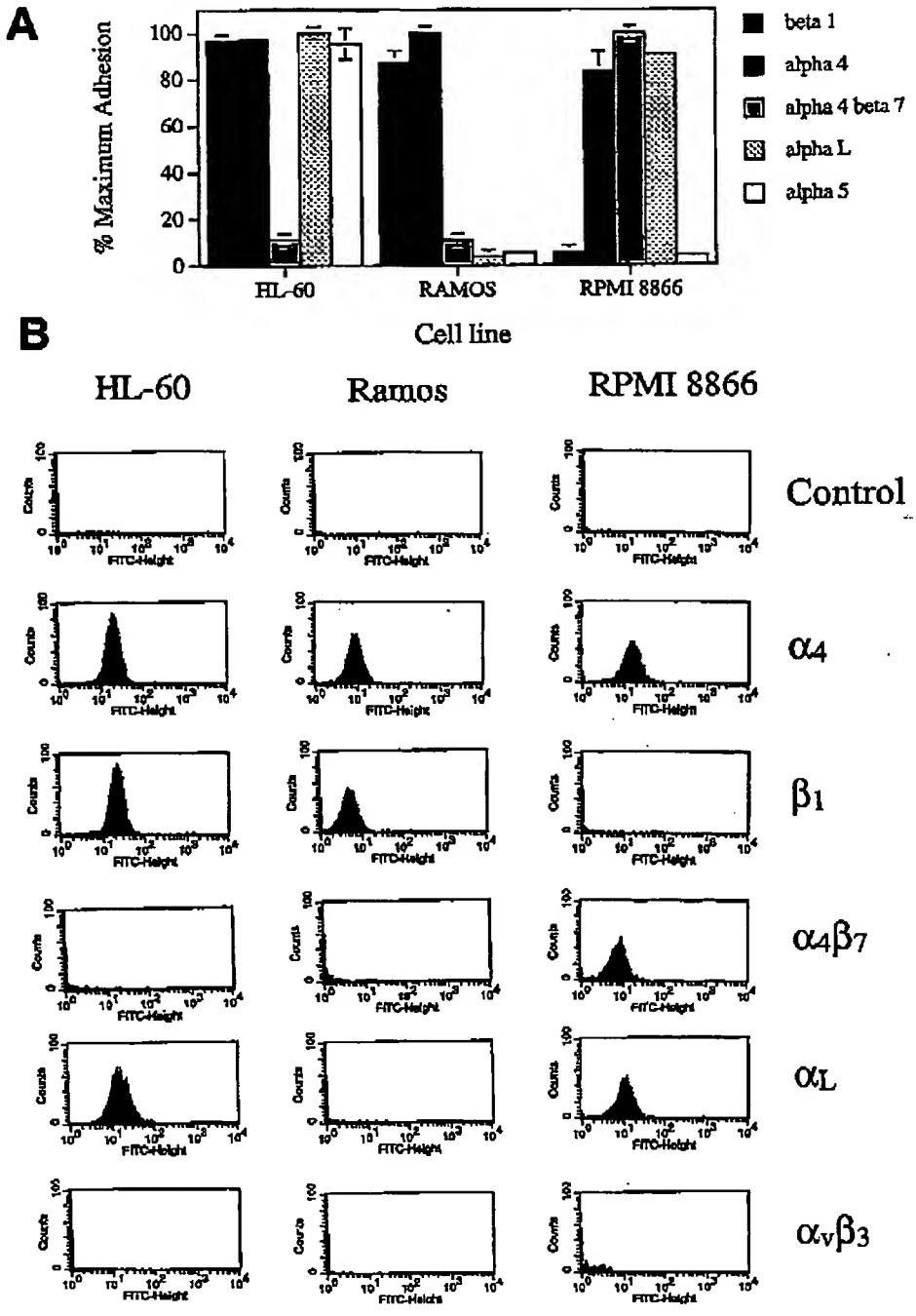

Fig. 2. Integrin expression by HL-60 and Ramos cells. (A) Leukocyte adhesion to anti-integrin monoclonal antibodies directed to α_4 (HP2/1), β_1 (Mab$_{13}$), $\alpha_4\beta_7$ (Fib 504), α_L (TS1/22) and α_5 (Mab$_{16}$). HL-60, Ramos and RPMI 8866 cells were added to microwells coated with 5 μg/ml antibodies in the absence of cations and allowed to adhere for 30 minutes and quantitated as described in Materials and Methods. Data shown is a representative experiment (n=3) performed in triplicate and values shown are mean absorbance (± s.e.m.). (B) FACS analysis of integrin expression. HL-60, Ramos and RPMI 8866 cells were incubated with 5 μg of anti-integrin monoclonal antibodies directed to α_4 (HP2/1), β_1 (Mab13), $\alpha_4\beta_7$ (Fib 504), α_L (TS1/22), and $\alpha_v\beta_3$ (LM 609). The cells were washed, incubated with fluorescein-conjugated goat anti-mouse IgG, washed again and then analyzed using FACS.

peptide is a competitive inhibitor of $\alpha_4\beta_1$ integrin binding to fibronectin (Komoriya et al., 1991). The RGD peptide is a known inhibitor of cell attachment occurring through RGD sites (Pierschbacher and Ruoslahti, 1984). The LEV and RGE peptides serve as inactive controls. As shown in Fig. 3B, the LDV peptide completely abolished adhesion to OPN compared to control, while LEV and RGE had no effect. An RGD peptide showed slight inhibitory activity. Previous work has indicated that RGD peptides can bind $\alpha_4\beta_1$ under some conditions of leukocyte activation and block its function (Sanchez-Aparicio et al., 1994). The antibody and peptide data combined show that $\alpha_4\beta_1$ mediates leukocyte adhesion to OPN.

To ensure that leukocyte adhesion to OPN observed in the presence of different levels of Ca^{2+} and Mg^{2+} (Fig. 1) occurred through $\alpha_4\beta_1$, separate blocking experiments were performed in the presence of varying cation levels. Fig. 5 illustrates that

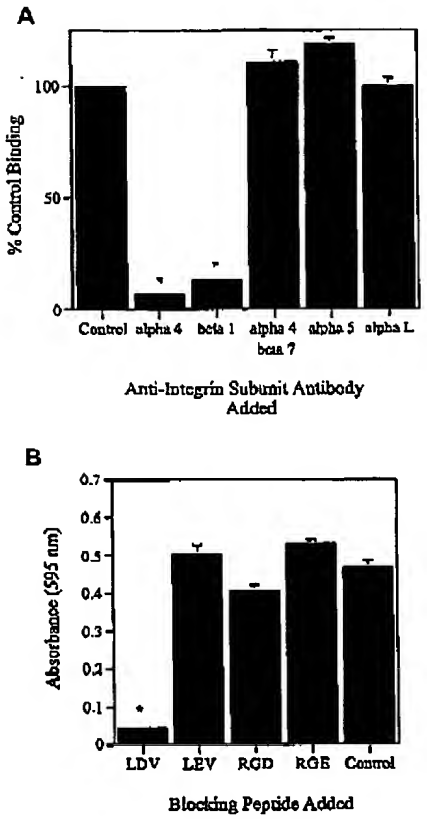

Fig. 3. Leukocyte adhesion experiments indicating that the $\alpha_4\beta_1$ integrin is responsible for HL-60 cell adhesion to OPN. (A) Combined experiments where activated HL-60 cells were incubated with 20 μg/ml antibody for 15 minutes at 37°C prior to plating (n=4). Antibodies utilized included HP2/1 (α_4), Mab_{13} (β_1), Fib 504 ($\alpha_4\beta_7$), Mab_{16} (α_5) and TS1/22 (α_L). Control indicates no antibody present. Experiments were performed and quantitated as described in Materials and Methods. Values represent the absorbance readings averaged from two experiments in triplicate wells (± s.e.m.). *P<0.001 compared to control using Student's t-test. (B) Leukocyte adhesion experiments showing that LDV, an $\alpha_4\beta_1$-binding peptide completely inhibits OPN-mediated cell attachment (n=4). Activated HL-60 cells were incubated with 250 μg/ml peptide in the presence of 3 mM Mg^{2+} for 15 minutes at 37°C prior to plating. LEV and RGE peptides are inactive and serve as controls. Control indicates no peptide present. Experiments were performed and quantitated as described in Materials and Methods. Values represent mean absorbance readings from a representative experiment performed in triplicate wells (± s.e.m.). *P<0.001, †P<0.025 compared to control using Student's t-test.

the LDV peptide markedly blocked HL-60 cell adhesion in the presence of Ca^{2+} and Mg^{2+} or with either Ca^{2+} or Mg^{2+} alone. In contrast, the LEV peptide showed no effect.

The $\alpha_4\beta_1$ integrin directly binds OPN

Further investigation showed that $\alpha_4\beta_1$ directly bound OPN in affinity chromatography experiments. HL-60 and Ramos cells were surface-labeled with biotin, extracted with detergent, and the extracts were incubated with OPN-Sepharose in the presence of 1.5 mM Mg^{2+} and 1.5 mM Mn^{2+}. After incubation, the columns were washed in the presence of divalent cations and eluted with EDTA (Fig. 6A). For the HL-60 cell extracts, integrins eluted in fractions 3 and 4, while for Ramos cell extracts, fractions 2 and 3 contained integrins. The molecular masses of these bands, 150 and 130 kDa, match that of the α_4 and β_1 subunits, respectively. To identify these integrins, immunoprecipitations were performed using anti-integrin antibodies (Fig. 6B). For both cell types, α_4 and β_1 subunits were observed in the immunoprecipitates. Three different anti-α_4 antibodies were capable of immunoprecipitating the band identified as the α_4 subunit (Fig. 6B, Ramos) while control antibodies did not. Interestingly, the $\alpha_5\beta_1$ integrin was also identified from the HL-60 cell experiment, suggesting it may have an affinity for OPN coupled to Sepharose, presumably through its RGD site. However, there is no evidence that $\alpha_5\beta_1$ participates in the adhesion of HL-60 cells to OPN based on our adhesion data where addition of anti-α_5 blocking antibodies had no effect on adhesion and RGD peptides showed minimal inhibitory effects. The interaction of $\alpha_5\beta_1$ with OPN-Sepharose may be related to the presence of Mn^{2+} in the buffers used during affinity chromatography. In contrast, the adhesion assays were

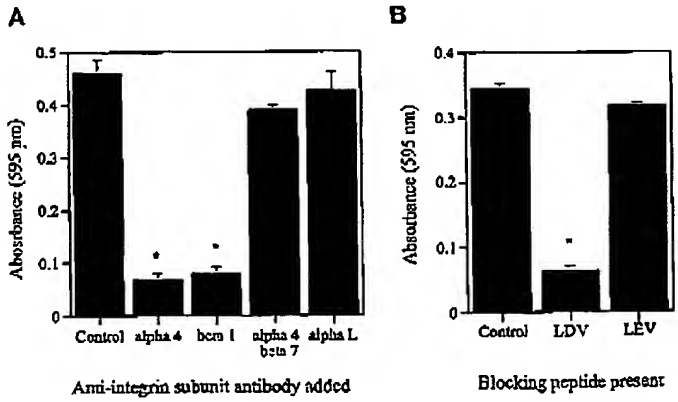

Fig. 4. Antibody and peptide blocking data indicating Ramos adhesion to OPN occurs through the $\alpha_4\beta_1$ integrin. (A) Ramos cells were incubated with 20 μg/ml antibody for 15 minutes at 37°C prior to the addition of 1 μg/ml 8A2. Cells were plated and binding quantified as described in Materials and Methods (n=3). Antibodies utilized included HP2/1 (α_4), Mab_{13} (β_1), Fib 504 ($\alpha_4\beta_7$), and TS1/22 (α_L). Control indicates no antibody present. Values represent the absorbance readings from a representative experiments in triplicate wells (± s.e.m.). (B) Ramos cells were activated with the monoclonal antibody 8A2 (1 μg/ml). After OPN adsorption to microwells, wells were blocked with 0.1% Tween-20 for at least 30 minutes followed by extensive washing of the wells with water. After rinsing, 2 mM Ca^{2+} and 1 mM Mg^{2+} were added prior to a 15 minute incubation at 37°C with 100 μg/ml of the LDV and LEV peptides along with 0.001% Tween-20 prior to plating. Experiments were performed and quantitated as described in Materials and Methods. The data shown are from representative experiments (n=3) performed in triplicate wells and values are shown as mean absorbance (± s.e.m.). *P<0.001 compared to control using Student's t-test.

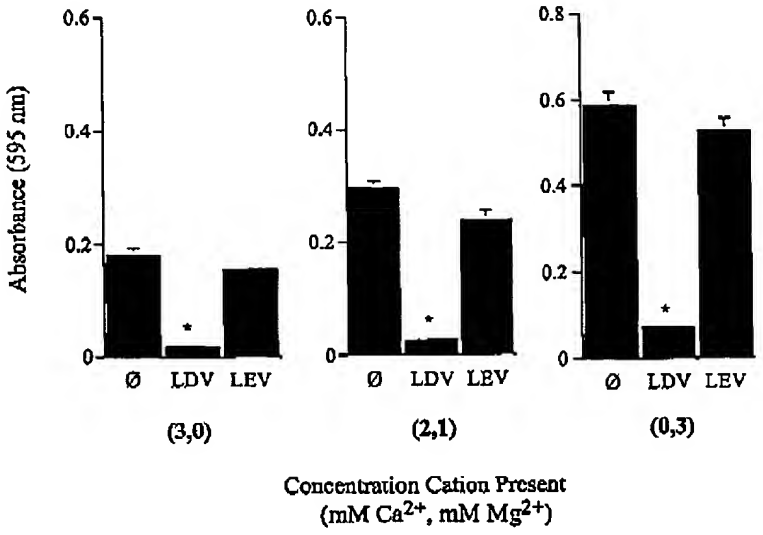

Fig. 5. The effects of LDV on leukocyte adhesion in the presence of various concentrations of Ca^{2+} and Mg^{2+}. Activated HL-60 cells were preincubated for 15 minutes at 37°C with both LDV and LEV peptides (250 μg/ml) and with various combinations of Mg^{2+} and Ca^{2+} before plating. For all groups, control (Ø) indicates the absence of peptide during incubation. Values represent actual absorbance readings from a representative experiment in triplicate wells (± s.e.m.). *P<0.001 compared to control using Student's t-test.

performed with Mg^{2+} and Ca^{2+} which may alter the ability of $\alpha_5\beta_1$ to bind.

An NH_2-terminal thrombin fragment of OPN promotes $\alpha_4\beta_1$-dependent HL-60 cell adhesion

As an initial step to locate the $\alpha_4\beta_1$-binding site within OPN, the native protein was proteolytically cleaved with thrombin. The fragments were tested for activity and found to promote dose-dependent adhesion of leukocytes comparable to the native protein (not shown). Separation of fragments was accomplished using fast protein liquid chromatography (FPLC) and anion exchange utilizing a Q-Sepharose column. This experiment was performed in the presence of 6 M urea to prevent the fragments from aggregating. SDS-PAGE analysis of eluted fractions is shown in Fig. 7A. Copper staining of the elution profile revealed a single 30 kDa band in fractions 15

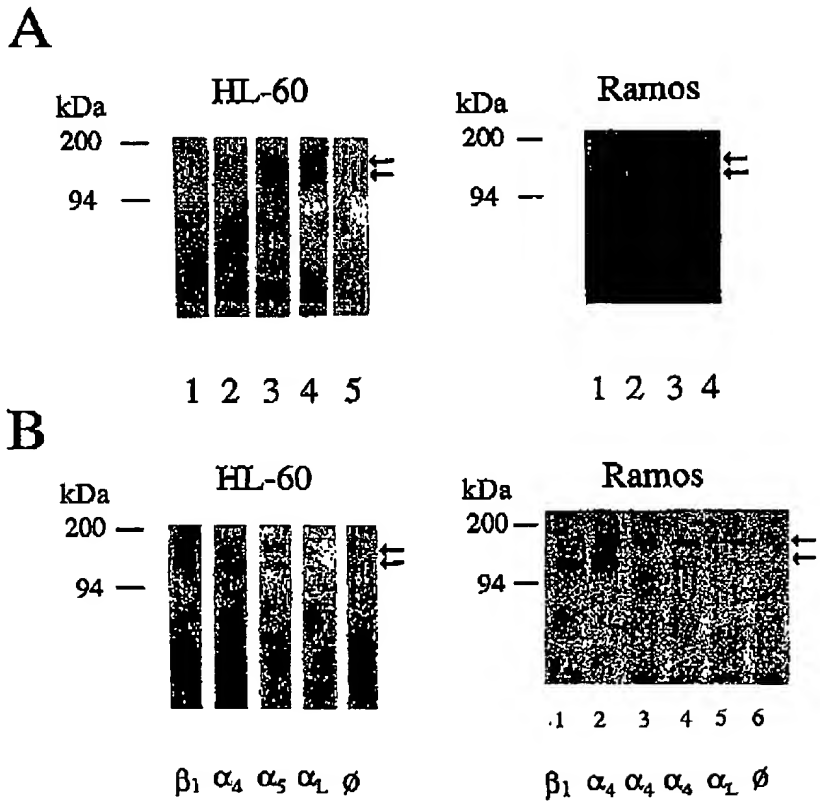

Fig. 6. Affinity chromatography data illustrating the binding of $\alpha_4\beta_1$ to OPN-Sepharose. (A) OPN-Sepharose was incubated with labeled HL-60 and Ramos cell lysates as described in Materials and Methods. 0.5 ml EDTA elution fractions were collected, run on SDS-PAGE gels and blots were developed. Left: HL-60 cell integrin elution pattern is shown (lanes 1-5). Right: Ramos cell integrin elution pattern (lanes 1-4). Upper arrows denote the α subunit, while lower arrows denote the β subunit. (B) Immunoprecipitations were performed for both cell types as described in Materials and Methods. Monoclonal or polyclonal antibodies directed to HL-60 and Ramos cell integrin subunits are indicated below each figure. Note that three different α_4-subunit specific antibodies were utilized in the Ramos cell immunoprecipitation experiment. These are α_4 subunit cytodomain, P4C2 and HP2/1 in lanes 2, 3 and 4, respectively. Upper arrows denote the α subunit, while lower arrows denote the β subunit.

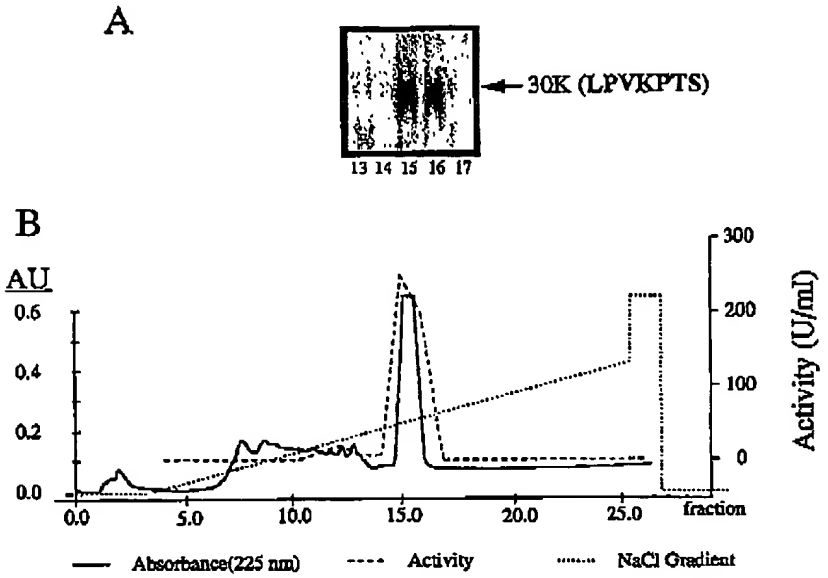

Fig. 7. Purification of a 30 kDa, NH_2-terminal fragment of OPN. OPN was cleaved using thrombin-Sepharose and injected onto a fast protein liquid chromatography column, Q-Sepharose in the presence of 6 M urea. A linear NaCl gradient (0-1.0 M) was performed over a 30 minute period (n=5). Fractions (600 μl) were collected and analyzed using SDS-PAGE, shown in A. Amino-terminal sequence analysis of the 30 kDa band revealed the sequence L-P-V-K-P-T-S, matching exactly the N terminus of bovine OPN. (B) Tracings indicating the elution profile, leukocyte adhesive activity and linear NaCl gradient for the Q-Sepharose column. To identify which fractions were active in promoting HL-60 cell adhesion, wells were coated with serial two-fold dilutions of each fraction and cell adhesion assays were performed with activated cells in the presence of 2 mM Ca^{2+}, 1 mM Mg^{2+}. The resulting activity units are expressed as U/ml where a unit corresponds to the dilution at which half-maximal adhesion occurred.

and 16. Fig. 7B shows the NaCl gradient utilized and the resulting elution and activity profiles. A single major peak consistently eluted at 0.5 M NaCl. Single fractions from the elution pattern shown were tested for their ability to promote cell adhesion. Although a small amount of activity was present in fractions 11-13 (Fig. 7B), by far the majority of the activity was seen in fractions 15-16, corresponding to the major elution peak. Amino-terminal sequence analysis of the 30 kDa fragment revealed LPVKPTS, matching the N terminus of bovine OPN. In addition, the 30 kDa fragment stained poorly with Coomassie blue and could only be visualized using a copper stain. These characteristics of the 30 kDa fragment are consistent with those previously reported for an N-terminal fragment of human OPN (Senger and Perruzzi, 1996). Although we did successfully purify an N-terminal fragment of bovine OPN, we were unable to isolate any defined C-terminal fragments in sufficient quantities. This may be due to heterogeneity in the thrombin proteolysis of the C-terminal region of OPN or to further proteolytic degradation of C-terminal fragments.

Dose-response curves illustrating cell adhesion for the N-terminal fragment versus native OPN are shown in Fig. 8. The efficacies of the two OPN preparations in promoting HL-60 cell adhesion were similar. Both species promoted dose-dependent adhesion of leukocytes in the presence of 2 mM Ca^{2+} and 1 mM Mg^{2+}. Native OPN and the N-terminal fragment also promoted dose-dependent adhesion of human endothelial cells (not shown). Additionally, leukocyte adhesion to the N-terminal fragment was blocked by LDV peptides but not by the control LEV peptide, indicating that adhesion occurred through the $\alpha_4\beta_1$ integrin (Fig. 8B).

DISCUSSION

We present here data showing that OPN is a new ligand for the $\alpha_4\beta_1$ integrin. This work was facilitated by the use of two leukocyte cell lines, HL-60 and Ramos, which do not express the $\alpha_v\beta_3$ integrin that recognizes the RGD site in OPN. Cell adhesion experiments identified $\alpha_4\beta_1$ as the receptor responsible for leukocyte adhesion to OPN. Affinity chromatography was used to show that the $\alpha_4\beta_1$ integrin from HL-60 and Ramos cells bound OPN-Sepharose. An isolated N-terminal thrombin fragment of OPN promoted adhesion of leukocytes in an $\alpha_4\beta_1$-dependent manner. These data collectively show that the $\alpha_4\beta_1$ integrin recognizes OPN and that at least one binding site for the integrin is present within the N-terminal region of the protein.

The $\alpha_4\beta_1$ integrin promotes leukocyte adhesion to osteopontin

In our system, leukocyte adhesion to OPN required cell activation. This was accomplished by the addition of TPA and the β_1-activating antibody, 8A2 for HL-60 cells while only 8A2 was necessary for the activation of Ramos cells. These findings are consistent with previous work showing that the activation state of $\alpha_4\beta_1$ is variable among different leukocyte cell lines and that different degrees of activating stimuli are necessary to induce $\alpha_4\beta_1$-mediated adhesive events (Masumoto and Hemler, 1993). Activation of leukocytes in the presence of Mg^{2+} and Ca^{2+} resulted in significant leukocyte adhesion to OPN as compared to non-activated cells. Leukocyte adhesion occurred in various combinations of Ca^{2+} and Mg^{2+} including physiological concentrations (2 mM Ca^{2+}, 1 mM Mg^{2+}).

Antibody and peptide inhibition experiments were performed to provide evidence that the $\alpha_4\beta_1$ integrin was responsible for leukocyte adhesion to OPN. Consistent with the participation of $\alpha_4\beta_1$, the addition of α_4 or β_1 blocking antibodies significantly inhibited HL-60 and Ramos cell adhesion to OPN. In peptide inhibition experiments, the $\alpha_4\beta_1$-specific peptide, LDV, completely abolished cell attachment compared to control for both cell types, while LEV, the control peptide, did not block adhesion. In addition, our affinity chromatography data show that OPN-Sepharose bound the

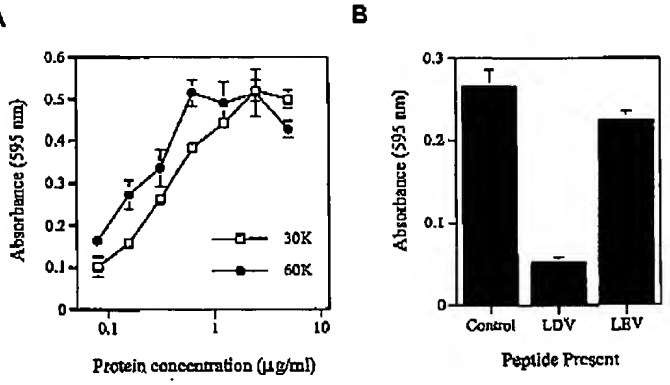

Fig. 8. The 30 kDa fragment of OPN promotes dose- and LDV-dependent adhesion of leukocytes. (A) Comparison of the ability of intact and 30 kDa OPN fragment to promote HL-60 cell adhesion. Concentrations of the 30 kDa fragment and intact OPN were determined using spectral analysis based on a method by Pace et al. (1995). Wells were coated with 5 μg/ml of each protein and serially diluted twofold. Activated cells were added in the presence of physiological cations and allowed to adhere for one hour. The data shown are from a representative experiment performed in triplicate wells and values are shown as mean absorbance (± s.e.m.). (B) Blockade of HL-60 adhesion to the 30 kDa fragment of OPN with the LDV peptide. Triplicate wells were coated with 5 μg/ml of the 30 kDa fragment and blocked with BSA. HL-60 cells were activated and incubated with the 20 μg/ml of the LDV and LEV peptides or nothing for 15 minutes prior to plating. Cells were allowed to adhere for one hour and quantitated as described in Materials and Methods. The data shown are the average of two experiments performed in triplicate wells. Values represent mean absorbance (± s.e.m.).

$\alpha_4\beta_1$ integrin from both HL-60 and Ramos cell lysates. These data suggest that $\alpha_4\beta_1$ binds OPN and mediates leukocyte adhesion to OPN.

Thrombin fragments of OPN were generated to investigate the location of $\alpha_4\beta_1$ binding sites in OPN. These fragments were loaded onto an FPLC ion-exchange column and one major peak eluting at 0.5 M NaCl was recovered. Copper staining of the elution profile revealed a single 30 kDa band corresponding to the major elution peak. Amino-terminal sequence analysis confirmed its identity as an NH_2-terminal fragment of OPN. Our data are consistent with the characteristics of the thrombin-generated N-terminal fragment of human OPN previously reported (Senger et al., 1996) in that staining was only accomplished using the copper staining technique and the fragment promoted endothelial cell adhesion. Here, we have shown that this OPN fragment promoted dose-dependent adhesion of leukocytes through $\alpha_4\beta_1$. Although we were successful in isolating an N-terminal OPN fragment, we were unable to isolate sufficient amounts of defined C-terminal fragments. We believe that thrombin digestion resulted in heterogeneous fragmentation of the C-terminus of OPN. While we cannot rule out the possibility that sequences within the C-terminal domain of OPN may facilitate leukocyte adhesion through $\alpha_4\beta_1$, the N-terminal domain promoted adhesion in an essentially identical manner to that of intact OPN, suggesting it contains an $\alpha_4\beta_1$ binding site. An important point is that there are no LDV, IDS or EDV sequences within this domain which are known $\alpha_4\beta_1$-binding sites from either fibronectin or VCAM-1 (Springer, 1994; Carlos and Harlan, 1994; Lobb and Hemler, 1994). Our peptide inhibition experiments with LDV and RGD peptides indicate the RGD site in OPN probably does not play a major role in the $\alpha_4\beta_1$-OPN interaction. Thus, the $\alpha_4\beta_1$-binding site within OPN will be a novel binding site, possibly related to LDV or IDS, and further work will be necessary to identify this site.

Relevance to vascular injury and inflammation

Ample evidence in the literature exists showing the expression of OPN following many types of injury. In granulation tissue and necrotic myocardium derived from either rat or human myocardial infarction, OPN mRNA and protein were found to be expressed at high levels (Murry et al., 1994). OPN was also found to be upregulated in cases of tubulointerstitial injury (Eddy et al., 1995; Giachelli et al., 1994) and glomerulonephritis (Pichler et al., 1994). OPN has been found to be heavily expressed in human atherosclerotic plaques (Giachelli et al., 1993; Hirota et al., 1993; Ikeda et al., 1993; O'Brien et al., 1994) and in neointimal cells of arteries injured by balloon angioplasty (Giachelli et al., 1991; Liaw et al., 1995a). As a result of these data, it has been proposed that OPN is a general marker for injury (Murry et al., 1994).

Recent work also indicates an intriguing overlap of OPN expression and the $\alpha_4\beta_1$ integrin following vascular injury. An increase in the expression of the $\alpha_4\beta_1$ integrin was observed in both smooth muscle cells of human atherosclerotic plaques and dedifferentiated VSMCs in vitro (Duplaa' et al., 1997). OPN expression has been shown to be induced under similar conditions (Giachelli et al., 1991, 1993; Hirota et al., 1993; Ikeda et al., 1993; O'Brien et al., 1994). Thus, the present findings showing the interaction between the $\alpha_4\beta_1$ integrin and OPN may have functional relevance for a variety of cell types in vascular injury or disease.

The function of OPN in pathophysiological states remains to be determined. The induction of OPN may serve as a recruiting stimulus for macrophages and monocytes to the injured area since subcutaneous injection of OPN resulted in a substantial increase in leukocytes within the injection site (Singh et al., 1990; Nasu et al., 1995). Several studies have demonstrated that OPN expression precedes monocyte/macrophage accumulation (Giachelli et al., 1994; Murry et al., 1994; Pichler et al., 1994; Eddy et al., 1995; Wiener et al., 1996). In addition, OPN may be an adhesive or immobilizing substrate for monocytes/macrophages based on evidence that OPN promotes adhesion of macrophages (Singh et al., 1990). OPN may also stimulate other macrophage functions such as phagocytosis or production of cytokines and proteolytic enzymes which are involved in wound repair responses.

In the work presented here, we provide evidence that leukocytes can utilize the $\alpha_4\beta_1$ integrin as a receptor for OPN. This $\alpha_4\beta_1$-OPN interaction may be relevant in a variety of cellular responses during tissue injury. This finding adds further support to the general concept that induction of OPN

following injury may be an important signal for cells involved in the modulation of inflammatory and tissue injury responses.

The authors thank Dr Nicholas Kovach and Dr Francisco Sanchez-Madrid for their kind gifts of 8A2 and HP2/1 antibodies, respectively. We thank Drs David Erle and Russell Pachynski, for providing the RPMI 8866 cell line. Also, we appreciate Eric Nicholson's help with FPLC. This work was supported by NIH grants HL 59373 (G.E.D.) and HL 33324 (G.A.M.).

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 13, Issue of March 28, pp. 8447–8453, 1997
Printed in U.S.A.

Propolypeptide of von Willebrand Factor Is a Novel Ligand for Very Late Antigen-4 Integrin*

(Received for publication, November 7, 1996)

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We have previously reported that propolypeptide of von Willebrand factor (pp-vWF) promotes melanoma cell adhesion in a β1 integrin-dependent manner. In this report, we identified the α subunit of the cell adhesion receptor for pp-vWF as α4. Human leukemia cell lines that express α4β1 integrin (very late antigen-4, VLA-4), but not cell lines which lack VLA-4, attached well to pp-vWF substrate and these adhesions were completely inhibited by anti-α4 integrin monoclonal antibody HP2/1. Adhesion of mouse melanoma expressing α4 integrin was also inhibited by anti-mouse α4 mAb PS/2. Furthermore, transfection of human α4 cDNA into α4⁻ Chinese hamster ovary cells resulted in an acquisition of adhesive activity to pp-vWF, indicating that pp-vWF is a ligand for VLA-4 integrin. Using a recombinant fragment of pp-vWF, the cell attachment site was shown to be located within amino acid residues 376–455 of pp-vWF. A series of synthetic peptides covering this region were tested for the ability to promote cell attachment and a 15-residue peptide designated T2-15 (DCQDHSF-SIVIETVQ, residues numbered 395–409) promoted VLA-4 dependent cell adhesion. The peptide was also capable of inhibiting cell adhesion to pp-vWF, suggesting that this sequence represents the cell attachment site. By affinity chromatography using peptide T2-15-Sepharose, it was found that α4β1 integrin complex from extracts of surface iodinated B16 cells specifically bound to the peptide. These results strongly suggest that pp-vWF is a novel physiological ligand for VLA-4.

Propolypeptide of von Willebrand factor (pp-vWF),[1] which is also called von Willebrand antigen II (1), is an unusually large propolypeptide (~100 kDa) produced only in endothelial cells and megakaryocytes together with blood coagulation protein von Willebrand factor (2). It is processed from a large precursor of vWF (prepro-vWF) during biosynthesis and stored in the granule of both endothelial cells and platelets independent from mature vWF (3, 4). We have been investigating the biological functions of pp-vWF and found that pp-vWF bound to collagen and inhibited collagen-induced platelet aggregation in contrast to the mature vWF (5, 6). Furthermore, we have found that pp-vWF serves as a substrate for transglutaminase and is specifically cross-linked to laminin (7), suggesting a possibility that it acts as transient matrix protein upon secretion from platelets and endothelial cells at the site of vascular injury. In a previous paper (8), we reported that pp-vWF promoted the attachment and spreading of melanoma cells. The receptor responsible for this adhesion was the β1 class of integrin but the corresponding α subunit could not be identified.

Integrins are heterodimeric transmembrane proteins consisting of α and β subunits and mediate cell adhesion to extracellular matrix proteins as well as cell-cell interactions (9–12). To date more than 15 α subunits and 8 β subunits have been identified and combination of α and β subunits determines the ligand specificity of individual integrins. Integrin-mediated cell adhesion plays crucial roles in regulating the morphology, proliferation, migration, and differentiation of cells. Ligands for integrins are quite diverse and include many extracellular matrix proteins as well as cell surface molecules. VLA-4 (α4β1) is an integrin complex that recognizes both alternatively spliced segment III (CS1) of fibronectin (13), and vascular cell adhesion molecule-1 (VCAM-1) (14). Lymphocyte adhesion to endothelial cells is primarily mediated by interaction of lymphocyte VLA-4 with VCAM-1 expressed on cytokine-activated endothelial cells and this pathway is thought to be central to the lymphocyte recruitment to the site of inflammation (15). Moreover, interactions of VLA-4 and its counter-receptors have also been implicated in a number of physiologic and pathogenic processes including CD3-dependent T cell activation (16), lymphohemopoiesis (17–19), myogenesis (20), and melanoma cell metastasis (21, 22). Therefore, VLA-4 is attracting broad attention from those who are investigating the molecular mechanisms underlining these processes.

In the present paper, we found that pp-vWF is the novel ligand for the VLA-4. Furthermore, we identified the cell attachment site as a 15-residue linear sequence present in the midregion of pp-vWF molecule. A synthetic peptide with this sequence could promote cell attachment in an α4β1 integrin-dependent manner and directly bound to VLA-4 complex.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibody (mAb) 4B4 recognizing human β1 integrin was a gift from Dr. C. Morimoto, Dana-Farber Cancer Institute, Boston, MA. MAbs A1A5 and TS2/16 (both anti-human β1) were obtained from Dr. M. E. Hemler (Dana-Farber Cancer Institute, Boston, MA). Mouse mAb 6F1 (anti-α2) was from Dr. B. S. Coller (State Uni-

* This work was supported in part by General Scientific Research Grant-in-Aid 06454644 from the Ministry of Education, Science, and Culture of Japan and by grants from the Ito Memorial Foundation, the Nissan Science Foundation, and the Kanagawa Academy of Science and Technology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: pp-vWF, propolypeptide of von Willebrand factor; CS1, type III connecting segment region 1; mAb, monoclonal antibody; CHO, Chinese hamster ovary; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis.

versity of New York, Stony Brook, NY), BIIG2 (anti-α5) was from Dr. C. Damsky (University of California San Francisco, San Francisco, CA). Mouse mAbs P1B5 (anti-α3), HP2/1 (anti-α4), and LM609(anti-αvβ3) were purchased from Terios Pharmaceutical Co. (La Jolla, CA), Cosmo Bio Co., Ltd (Tokyo Japan), and Chemicon International Inc. (Temecula, CA), respectively. A rat anti-α6 integrin mAb GoH3 was a gift from Dr. A. Sonnenberg, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands. Rabbit antisera to the C terminus of human α3B, α4, α5, and αv integrin subunits were kindly donated by Dr. E. Ruoslahti, La Jolla Cancer Research Institute, La Jolla, CA. Polyclonal antibody against C terminus of the α7 subunit was prepared by immunizing a synthetic peptide having sequence of cytoplasmic tail of human α7B subunit (DAHPILAADWH-PELG) in our laboratory. These polyclonal antisera all recognized respective subunits from mouse integrin because of the high interspecies conservation of the sequence in the cytoplasmic region. Rat hybridoma cells secreting anti-mouse α4 integrin PS/2 were obtained from American Type Culture Collection and ascites containing this antibody was produced. A recombinant VCAM-1-mouse Cκ chain fusion protein was donated by Dr. D. Dottavio (Sandoz Pharmaceuticals, East Hanover, NJ) and a CS1 peptide-rat serum albumin conjugate was a gift from Dr. E. Wayner (Fred Hutchinson Cancer Research Center, Seattle, WA). Synthetic peptides GRGDSP and DELPQLVTLPHPNLHGPEILDVPST (CS1) were purchased from Sigma. pp-vWF was purified from bovine-washed platelets by immunoaffinity chromatography as described previously (29).

Construction of Human Integrin Expression Vectors and Transfection—Wild-type human β1 integrin cDNA (24) was subcloned into pBJ-1 vector and transfected into Chinese hamster ovary (CHO) cells together with pFneo DNA containing the neomycin resistance gene by electroporation as described previously (25). Stable clones were selected using 700 μg/ml G418 (Life Technologies, Inc.), and cells expressing human β1 integrin most abundantly were selected by single-cell sorting using an anti-β1 mAb. For obtaining CHO cells expressing both human α4 and β1 integrins, wild-type human α4 cDNA (26) was transfected into β1 integrin-expressing clone together with CDhygro DNA containing the hygromycin-resistance gene and maintained in the medium containing 400 μg/ml hygromycin. Stable CHO cell line clones expressing high levels of human α4 were again selected by single-cell sorting using an anti-α4 mAb. Expression levels of each human integrin subunit were verified by fluorescence-activated cell sorting analysis using FACScan (Becton Dickinson, Mountain View, CA) according to the method described previously (24).

Cloning and Expression of Recombinant 8-kDa Fragment Variants—A cDNA clone encoding the 8-kDa portion of human pp-vWF was synthesized using reverse transcriptase-polymerase chain reaction amplification of human placental mRNA. Briefly, total mRNA was prepared using the guanidinium isothiocyanate/cesium chloride method (27). First strand cDNA was then generated by reverse transcription using a 3′-oligonucleotide primer complementary to the C terminus of the 8-kDa fragment (Lys^{455}) with a terminal *Eco*RI restriction sequence (5′-GAATTCTTTCAGGAGGGGGAGCTGGA-3′). The reverse transcribed cDNA mixture was subjected to 30 amplification cycles of polymerase chain reaction using a 5′ primer with an *Eco*RI restriction sequence (5′-GAATTCACTTCAAGAGCTTTGACAACAGATA-3′). The polymerase chain reaction products were ligated into the pBluescript and transformed into *Escherichia coli* JM109. The cloned cDNA was identified by restriction analysis and sequenced using the dideoxy chain termination method. The insert was then subcloned into the *Eco*RI site of a modified version of the pGEX-2T expression vector (Pharmacia, Milton Keynes, United Kingdom), which included additional cloning sites in its polylinker, and were used to transform JM109. Recombinant clone (pGEX-r8k1A) was checked for its insert orientation by restriction mapping. Using the same polymerase chain reaction product as a starting material, two additional clones containing an insert of the truncated version of the 8-kDa fragment (r8k1B, corresponds to Ser^{272}-Asp^{415}, and r8k2A, corresponds to Gln^{409}-Lys^{455}) were also obtained. Primers used in the amplification of r8k1B were antisense primer 5′-GAATTCTGC-AGTCAGTCGCGGTCATCAGCACACT-3′ and sense primer 5′-GAAT-TCACTTCAAGAGCTTTGACAACAGATA-3′; primers for amplification of r8k2A were antisense primer 5′-GAATTCTTTCAGGAGGGGGAGC-TGGA-3′ and sense primer 5′-GAATTCTGCAGAAACAGTGTGCTGA-TGACCGCGA-3′.

Glutathione *S*-transferase (GST)-r8k1A fusion protein was induced and isolated as follows. Briefly, 5 ml of overnight cultures of JM109 transformed with recombinant plasmids were diluted 1:100 with fresh LB medium containing 50 μg/ml ampicillin and cultured for about 4 h at 37 °C. Isopropyl-β-D-thiogalactoside was then added to 0.5 mM and the culture continued for an additional 2 h. Cells were then centrifuged, suspended in 1/30 volume of phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride, and sonicated. As the fusion protein was completely recovered in the insoluble fraction, extracts were centrifuged and the pellet solubilized in 20 mM Tris-HCl, pH 8.0, containing 8 M urea and 0.2 mM β-mercaptoethanol, separated on a gel filtration column of Sephacryl S-200 equilibrated with the same buffer, and dialyzed against 20 mM Tris-HCl, pH 8.0, containing 1 M urea and 0.2 mM β-mercaptoethanol. The GST portion of the fusion protein was cleaved by the addition of 5 units/ml bovine thrombin (Sigma) for 14 h at room temperature. The cleavage mixture was then applied to a gel filtration column of Sephacryl S-200, and the cleaved 8-kDa fragment was further purified by reverse phase high performance liquid chromatography on a RESOURCE RPC column (Pharmacia). Fusion proteins of the truncated version of the 8-kDa fragment (r8k1B and r8k2A) were recovered in the soluble fraction and purified by glutathione-agarose affinity chromatography. Those proteins were pooled and dialyzed against 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, and stored at −70 °C. Protein concentrations were measured using the BCA assay (Pierce).

Peptide Synthesis—A series of 20-residue peptides (T1 to T5), which correspond to parts of the 8-kDa fragment, were synthesized by a multiple peptide synthesizer (Model 396, Advanced ChemTech Inc., Louisville, KY) using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry. Shorter peptides (T2–15, T2–10, T1-8 and BP-5) were synthesized using an Applied Biosystems 430A peptide synthesizer with *t*-butoxycarbonyl chemistry. In both cases, peptides were purified by high performance liquid chromatography and verified by fast atom bombardment-mass spectrometry as described previously (28). Peptides (4 mg) were covalently coupled to 2 ml of packed beads of CNBr-activated Sepharose 4B (Pharmacia Biotech Inc.) according to the manufacturer's instruction.

Cell Adhesion Assay—B16 murine melanoma, human monocytic cell lines U937 and THP-1, human lymphoma cell lines MOLT-3 and Jurkat, and human erythroleukemic cell line K562 were provided from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and cultured in either Eagle's minimum essential medium containing 10% fetal calf serum or RPMI 1640 medium containing 10% fetal calf serum and non-essential amino acids. Adherent cells were grown to near confluency and harvested by incubation with phosphate-buffered saline, pH 7.2, containing 2.5 mM EDTA and 2 mg/ml bovine serum albumin for 30 min at 37 °C. Detached cells as well as suspension cells were washed three times and suspended in serum-free Eagle's minimum essential medium or RPMI 1640 prior to the adhesion assay. In the case of assay using human leukemia cells and CHO cells transfected with human integrins, the cells were pretreated with 1:1500 dilution of anti-human β1 activating mAb TS2/16 ascites to activate human β1 integrin. Cell adhesion assay was performed according to the method described previously (8) with a slight modification. In brief, 6-mm square chips cut from bacteriologic plastic dishes (Falcon 1029) were coated for 16 h at 4 °C with 50 μl of bovine pp-vWF (5 μg/ml), CS1-rat serum albumin (0.1 μg/ml), and bovine fibronectin (10 μg/ml). Some peptides were coated by mounting peptide solution on the chips and drying up at room temperature. It was confirmed by the protein assay that more than 80% of ligands were absorbed on the surface under these conditions. For VCAM-1, chips were first coated with anti-mouse Cκ chain (2 μg/ml, Caltag Laboratories, South San Francisco, CA) and then incubated with recombinant VCAM-1-mouse Cκ chain (2 nmol/chip). After blocking nonspecific protein-binding sites by incubation with 10 mM Tris-HCl, 150 mM NaCl, pH 7.4, containing 1% bovine serum albumin and 100 μg/ml mouse IgG at room temperature for 1 h, the chips were placed at the bottom of 48-well tissue culture dishes (Costar 3548) and overlaid with $2–5 \times 10^5$ cells in 50 μl of serum-free medium. After incubation at 37 °C for 90 min, the chips were picked up and rinsed in cold phosphate-buffered saline to remove nonadherent cells, and fixed with 1% glutaraldahyde in phosphate-buffered saline. Adherent cells were either photographed or counted using a light microscope with a calibrated grid marked on the ocular lens. An adhesion assay using peptide-coupled Sepharose was conducted as follows. Cell suspension was added to 96-well microtiter plate containing monolayer of peptide-Sepharose beads and incubated at 37 °C for 90 min. The beads bearing adherent cells were fixed with 1% glutaraldehyde in phosphate-buffered saline and separated from nonadherent cells by differential centrifugation, and stained with Giemsa prior to observation.

Affinity Chromatography—Confluent B16 melanoma cells grown in a 100-mm dish were detached as described above and iodinated by the lactoperoxidase/glucose oxidase method (29). Cells were then lysed with 1 ml of 100 mM octyl-β-D-glucopyranoside in 10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 2.5 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM phenyl-

TABLE I
Adhesion of hematopoietic cells to pp-vWF

Cells	Adhesion to pp-vWF	Integrin expression[a]					
		α2	α3	α4	α5	α6	αvβ3
THP-1	+	++	−	++	+	−	−
MOLT-3	+	−	±	++	−	±	−
Jurkat	+	+	+	++	+	+	−
U937	+	+	+	++	+	+	−
K562	−	+	−	−	+	±	±

[a] Expression of each integrin subunit was analyzed by FACS and scored according to the relative fluorescence intensity. −, no detectable fluorescence above background; ±, less than 2-fold increase in mean fluorescence channel; +, more than 2-fold but less than 20-fold increase in mean fluorescence channel; ++, more than 20-fold increase in mean fluorescence channel. The antibodies used are 6F1(α2), P1B5(α3), HP2/1(α4), BIIG2(α5), GoH3(α6), and LM609(αvβ3).

TABLE II
Effect of anti-α4 mAb on the cell adhesion to pp-vWF

Cells	Antibody	Specificity	Adhesion[a]
			% of control ± S.D.
MOLT-3	Control		100
	4B4	Human β1	0 ± 0
	HP2/1	Human α4	1 ± 0
	BIIG2	Human α5	93 ± 4
Jurkat	Control		100
	HP2/1	Human α4	2 ± 1
B16	Control		100
	PS/2	Mouse α4	0 ± 0

[a] Adhesions of the cells to pp-vWF were expressed as percent of the control that was conducted in the absence of antibodies. Data are mean ± S.E. from three independent experiments in which quadruplicate determinations were made. The concentrations of the antibodies were 5 μg/ml for purified antibody (4B4, HP2/1, and BIIG2) and 1:1000 dilution for ascites (PS/2).

methylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A at 4 °C for 15 min. After removal of insoluble material by centrifugation, the extract was mixed with an equal volume of peptide-Sepharose, gently shaken at room temperature for 3 h, and packed into a column. After washing out the unbound materials with 8 column volumes of a washing buffer (50 mM octyl-β-D-glucopyranoside, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2.5 mM $MgCl_2$, 1 mM $MnCl_2$) followed by 1 column volume of a pre-elution buffer (same as washing buffer without $MgCl_2$ and $MnCl_2$), the bound materials were eluted with an elution buffer which contains 5 mM EDTA in the pre-elution buffer. Eluted materials were collected at 1 ml/fraction and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gel under reducing conditions as described by Laemmli (30) followed by autoradiography using Bioimaging Analyzer BAS 2000 system (Fuji Film Co., Tokyo, Japan).

Immunoprecipitation—Eluates from T2–15-Sepharose were subjected to immunoprecipitation assay using anti-integrin antibodies. Samples were incubated either with 3 μl of antisera or 1 μg of IgG. Protein G/Protein A-agarose (Oncogene Science, Inc.) was then added to the mixture and incubated at 4 °C for 4 h with shaking. The immunocomplexes were centrifuged and the beads were washed four times with washing buffer as described above. The samples were then boiled in reducing Laemmli sample buffer and analyzed by SDS-PAGE on a 7.5% polyacrylamide gel as described above.

RESULTS

pp-vWF Serves as an Adhesion Substrate for α4β1 Integrin-expressing Leukemia Cells—In a previous study (8), we have found that only a limited number of cell lines are capable of adhering to pp-vWF, *i.e.* only two cell lines of melanoma origin out of more than 15 cell lines of both normal and tumor tissue origin adhered well to pp-vWF. Therefore, we thought at first that the receptor for pp-vWF is rather specifically expressed on melanoma cells. However, this was not the case because several cells of leukemic origin adhered to pp-vWF (Table I). Two monocytic cell lines, THP-1 and U937, as well as two lymphocyte-like cells, Jurkat and MOLT-3, are capable of attaching to the pp-vWF substrate in the presence of a human β1 integrin-activating monoclonal antibody (mAb) TS2/16. In contrast, an erythroleukemic cell line K562, which also expresses β1 integrin, did not adhere to pp-vWF even in the presence of both TS2/16 and Mn^{2+} ion. It is therefore very likely that the former four cell lines express integrin α subunit responsible for adhesion to pp-vWF while K562 does not. When the expression of various α subunit of integrin was analyzed by fluorescence-activated cell sorting analysis, the adhesive property of these cells corresponded well to the expression level of α4 integrin (Table I). As it is known that several melanoma cells also express high levels of α4β1 integrin, it is suggested that the α subunit of the β1 integrin receptor for pp-vWF is α4. To confirm this, the effect of anti-α4 integrin mAbs on the cell adhesion to pp-vWF was assessed. As depicted in Table II, adhesion of human leukemia cells to pp-vWF was completely inhibited by an anti-human α4 mAb HP2/1, as well as an anti-human β1 mAb 4B4, but not by an anti-human α5 mAb BIIG2. None of the function-blocking antibodies against α2, α3, and α6 subunit affected this adhesion (data not shown). The adhesion of mouse melanoma B16 was also inhibited by an anti-mouse α4 mAb PS/2, indicating that VLA-4 integrin is the responsible adhesion receptor for pp-vWF on both melanoma and hematopoietic cells.

Adhesion of α4β1 Integrin Transfectant to pp-vWF—To confirm that pp-vWF is a ligand for VLA-4 integrin, CHO cells were transfected with cDNA coding for human integrin subunits and the resultant stable transfectants were assessed for the ability to adhere to pp-vWF. As shown in Fig. 1A, the transfectant clones (β1-CHO and α4β1-CHO) express high levels of respective human integrin subunits. Both transfectants adhered well to plasma fibronectin (Fig. 1B), probably by using intrinsic α5 subunit complexed with both intrinsic and transfected β1 subunit. On the other hand, only α4β1-CHO cells adhered to CS1 and VCAM-1, the known ligands for VLA-4, indicating that they express functional VLA-4 integrin. Furthermore, α4β1-CHO but not β1-CHO adhered to pp-vWF. This adhesion of α4β1-CHO to pp-vWF was completely inhibited by an anti-α4 mAb HP2/1 (data not shown). These results strongly indicate that pp-vWF is a ligand for VLA-4 integrin.

Cell Adhesion Activity of Recombinant 8-kDa Fragment—As pp-vWF does not contain any homologous sequence to the known VLA-4 ligand sequences (CS1 region in fibronectin and first and fourth Ig domain in VCAM-1), we were interested in knowing what sequence in the pp-vWF primary structure is involved in cell adhesion. In a previous paper (8), we have already suggested that the cell adhesion site in the pp-vWF molecule resides within the central region of about 8 kDa. We have shown that an 8-kDa fragment generated by the lysylendopeptidase digest of bovine pp-vWF promotes melanoma cell adhesion in a dose-dependent manner. Furthermore, a mAb reactive with this fragment blocked the adhesive activity of pp-vWF. To determine the VLA-4 ligand sequence in the pp-vWF, we decided to construct a recombinant protein corresponding to this region. As amino acid sequence analysis of the isolated fragment suggests that it corresponds to a fragment having Ser^{378} as the N terminus and extends to Lys^{435} or Lys^{466}, we prepared a recombinant fragment containing sequence 373–455 in human pp-vWF (r8k1A, Fig. 2) using a conventional bacterial fusion protein expression system. When coated on plastic surface, this recombinant fragment did promote attachment and spreading of B16 melanoma (Fig. 3). When compared at molar basis, the fragment showed similar coating concentration dependence as intact pp-vWF protein, suggesting that this fragment contains the authentic cell attachment site. To narrow down the location of the active site, we prepared other clones expressing the N-terminal (r8k1B) and C-terminal (r8k2A) half of the 8-kDa fragment (Fig. 2) and

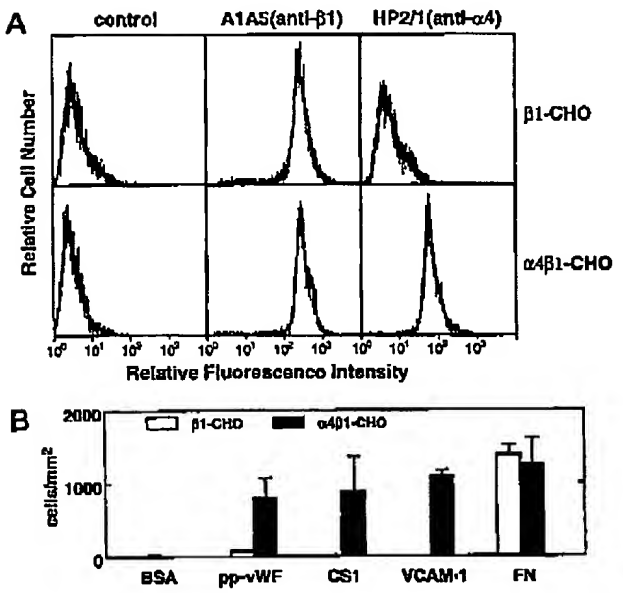

FIG. 1. **Transfection of α4β1 but not β1 integrin to CHO cells resulted in acquisition of adhesive activity toward pp-vWF.** *A*, fluorescence-activated cell sorting analysis of human integrin expression on transfectants. Stable clones from CHO cells transfected with either β1 alone or both α4 and β1 integrin cDNAs were checked for their expression of corresponding integrin subunit. Cells were stained with control mouse IgG (*control*), anti-human β1 mAb A1A5, or anti-human α4 mAb HP2/1. *B*, adhesive activity of each transfectant to various substrate. Established clone of β1-CHO (*open column*) or α4β1-CHO (*closed column*) were subjected to cell adhesion assay in the presence of β1-activating antibody TS2/16 as described under "Experimental Procedures." *FN*, fibronectin; *BSA*, bovine serum albumin.

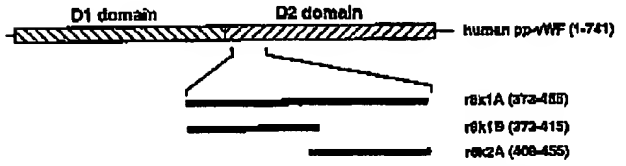

FIG. 2. **Schematic diagram of recombinant proteins contained in the central region of human pp-vWF molecule.** The *top part* of the figure shows the structure of the full-length pp-vWF subunit composed of two tandem repeats of D-type domains. The locations of amino acid sequences expressed as glutathione *S*-transferase fusion proteins are shown in *bold bars*. *Numbers* in parentheses indicate positions of amino acid residues in human pp-vWF sequence (3).

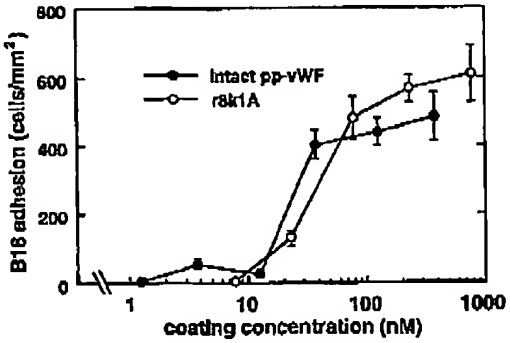

FIG. 3. **Attachment of B16 mouse melanoma cells to recombinant 8-kDa protein.** Adhesion activity of the recombinant 8-kDa fragment (r8k1A, *open circles*) is compared with that of intact pp-vWF (*closed circles*). Cell adhesion assay was carried out as described under "Experimental Procedures." The *abscissa* shows the coating concentration of the different proteins. Data are mean ± S.E. of one of the representative experiments in which triplicate determinations were made.

FIG. 4. **Attachment and spreading of B16 melanoma cells to the N-terminal half of the recombinant 8-kDa fragment.** GST fusion proteins bearing either the N-terminal (r8k1B) or C-terminal (r8k2A) half of the cell adhesive 8-kDa fragment were tested for their ability to support B16 melanoma adhesion. *Bar*, 100 μm.

assessed the cell adhesion activity of GST fusion protein of these peptides. As depicted in Fig. 4, B16 melanoma cells adhered to GST-r8k1B but not to GST-r8k2A. Again the extent of cell adhesion to the recombinant fragment (r8k1B) was comparable to that of intact pp-vWF (more than 80% of the input cells were adhered) indicating that the active site was solely located in the N-terminal half 42-residue portion.

Cell Adhesion Activity of Synthetic Peptides Derived from the 8-kDa Fragment—To further narrow down the cell attachment site in the 8-kDa region, we synthesized a series of peptides corresponding to this region. Five 20-residue peptides with overlapping sequence covering the entire length of the 8-kDa fragment were synthesized (Table III) and tested for their ability to support B16 mouse melanoma cell adhesion. Among the 5 peptides, 3 were active in supporting cell attachment (Fig. 5). However, the adhesions to peptides designated T1 and T4 were completely reversed by the addition of heparin. On the other hand, the adhesion to peptide T2 was not affected by heparin at all and, furthermore, was blocked by treatment of cells with an anti-mouse α4 integrin mAb, which was also observed with the intact pp-vWF. It can be concluded that the integrin recognition sequence is contained in this 20-residue portion of pp-vWF (391–410). As adhesion to intact pp-vWF was not affected by heparin at all, the cell attachment to T1 and T4 peptides could be a result of nonspecific electrostatic interaction between the cell surface and peptides, which was not exhibited when these sequences are included in the intact protein.

To obtain shorter peptides with the cell adhesion activity, we synthesized truncated versions of T2 peptide. As it was expected that shorter peptides might have problem in coating on the plastic surface by ordinary protocol, we immobilized these peptides covalently on Sepharose beads and performed a cell adhesion assay using the peptide-conjugated beads. As clearly depicted in Fig. 6, both 15- and 10-residue truncated peptides (T2–15 and T2–10, respectively) were active in supporting adhesion of B16 murine melanoma. A peptide derived from the central portion of T1 (T1-8) did not show any adhesive property under this condition. Although the extent of adhesion (average number of adherent cells on beads) was almost the same for T2–15 and T2–10, cell spreading on T2–10-Sepharose was less obvious than T2–15, suggesting that the 15-residue extension is necessary for full activity.

Inhibition of the Cell Adhesion to pp-vWF by Soluble Peptides—When the effect of soluble peptides on the B16 cell adhesion to pp-vWF was assessed, the CS1 peptide strongly inhibited the adhesion (Fig. 7). Complete inhibition was achieved at 30 μM. In contrast, a peptide containing RGD sequence did not affect adhesion at all, even at concentrations as high as 1 mM. Both peptide T2–15 and T2–10 had an inhib-

TABLE III
Synthetic peptides used in this study

Peptide	Amino acid sequence	Location	*M*	Net charge[a]	Hydrophilicity[b]
T1	SFDNRYFTFSGICQYLLARD	376–395	2416.7	0	−9.5
T2	LLARDCQDHSFSIVIETVQC	391–410	2277.6	−2	7.1
T3	ETVQCADDRDAVCTRSVTVR	406–425	2224.4	−1	−8.5
T4	SVTVRLPGLHNSLVKLKHGA	421–440	2126.5	+3	2.7
T5	LKHGAGVAMDGQDVQLPLLK	436–455	2090.5	0	1.3
T1–8	FSGICQYL	384–391	930.1	0	7.6
T2–15	DCQDHSFSIVIETVQ	395–409	1720.9	−3	−0.8
T2–10	DCQDHSFSIV	395–404	1150.2	−2	−1.3
BP-5	LEGCFCPPGLFLDENGSCHPK	662–682	2263.5	−2	−9.4

[a] Net charge is calculated by assuming a +1 net charge for Lys and Arg residues, and a net −1 charge for Glu and Asp at neutral pH. His is assumed to be uncharged at this pH.
[b] Calculated by the method of Kyte and Doolittle (45). According to this method, more hydrophilic peptides corresponds to the more negative values.

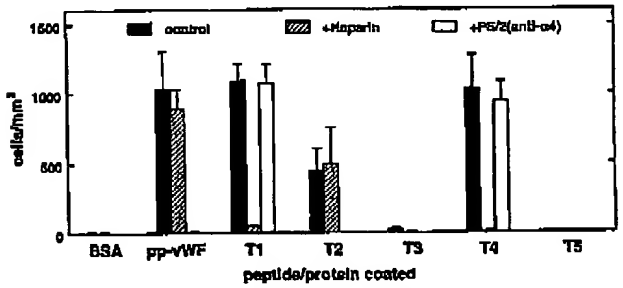

FIG. 5. **Properties of cell adhesion activity of synthetic peptides derived from the 8-kDa portion of human pp-vWF.** Five synthetic peptides were subjected to cell adhesion assay using B16 mouse melanoma in the absence (*closed column*) or presence of 1:1000 dilution of anti-mouse α4 integrin mAb PS/2 ascites (*open column*) or 100 μg/ml heparin (*hatched column*). Note that only the peptide designated T2 shows α4 integrin-dependent, heparin-independent adhesion like intact pp-vWF, while peptides T1 and T4 exhibit an α4 integrin-independent, heparin-inhibitable one. Data are mean ± S.E. of one of the representative experiments in which triplicate determinations were made. *BSA*, bovine serum albumin.

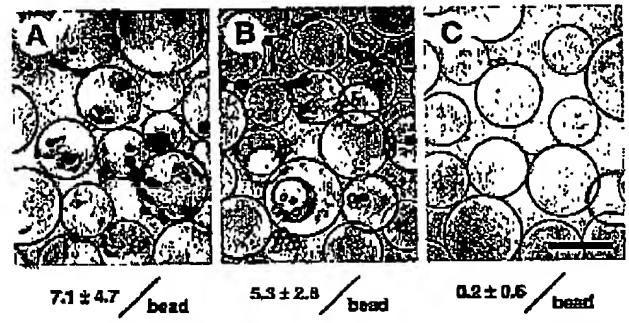

FIG. 6. **Attachment of B16 melanoma cells to Sepharose beads carrying peptides derived from the 8-kDa fragment.** Peptides T2–15 (*A*), T2–10 (*B*), and T1-8 (*C*) were coupled to CNBr-activated Sepharose and B16 melanoma cells were allowed to adhere on the beads, fixed, and photographed under a microscope. The *numbers under* each photograph denote the mean ± S.E. of the number of cells adhered per bead by counting more than 10 beads. *Bar*, 100 μm.

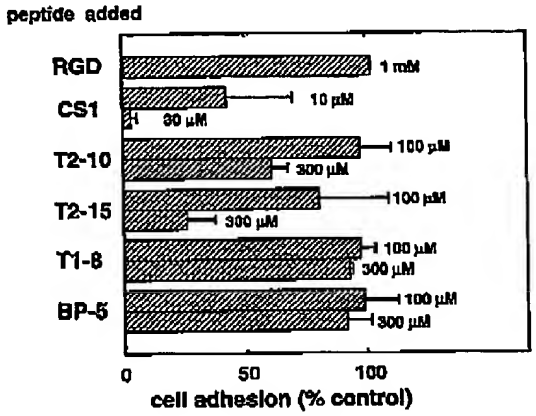

FIG. 7. **Inhibition of B16 cell adhesion to pp-vWF by the peptides.** Adhesion of B16 mouse melanoma cells to pp-vWF were assessed in the presence of the indicated concentration of peptides. Cells adhered were counted and the adhesion was expressed as percent of the number of cells adhered in the absence of peptide. The data represent mean ± S.E. of the three independent experiments in which triplicate determinations were made.

itory effect on the B16 adhesion to pp-vWF. When added at 300 μM, T2–15 inhibited cell adhesion by 75%. T2–10 had lower inhibitory activity compared with T2–15; the inhibition at 300 μM was only 40%, suggesting that the affinity of T2–10 is significantly lower than that of T2–15. Two control peptides, T1-8 and BP-5 (derived from a distant region of pp-vWF sequence containing similar net charge and hydrophilicity value), had essentially no inhibitory effect. Similar inhibition by CS1, T2–15, and T2–10 was also observed with the B16 cell adhesion to CS1-rat serum albumin but not with adhesions to collagen and laminin (data not shown), suggesting that the effect of these peptides is specific to VLA-4-mediated adhesion.

Binding of VLA-4 Integrin to T2–15 Peptide Immobilized on Sepharose Beads—The results presented above strongly indicate that the T2–15 sequence (DCQDHSFSIVIETVQ) represents the VLA-4-binding site in the pp-vWF molecule. To confirm this, binding of VLA-4 integrin to the T2–15 peptide was evaluated. As B16 murine melanoma cells adhered on T2–15-Sepharose (Fig. 6), it is obvious that the peptide retains active conformation to recognize its receptor even on the Sepharose beads. A control peptide derived from the central portion of T1 (T1-8) did not show any adhesive property under this condition. These peptide-Sepharose conjugates were then used as affinity matrices to isolate the cell surface receptor. Octyl-β-D-glucopyranoside extract of surface-iodinated B16 mouse melanoma was applied to an affinity column of T2–15-Sepharose, unbound materials were removed by washing, and the bound materials were eluted with EDTA. As shown in Fig. 8*A*, EDTA eluted two polypeptides of approximate molecular sizes of 150 and 125 kDa in the early step of elution from T2–15-Sepharose, followed by the elution of a 210-kDa protein in a rather retarded fraction. This 210-kDa band was also seen in the eluted fraction from a control column, T1-8-Sepharose, suggesting that it is a nonspecifically bound protein. The apparent molecular mass of the two polypeptides shifted from 150/125 kDa to 145/115 kDa when analyzed under nonreducing conditions (data not shown). These values are quite similar to that of the murine VLA-4 complex. To confirm that the bound material is

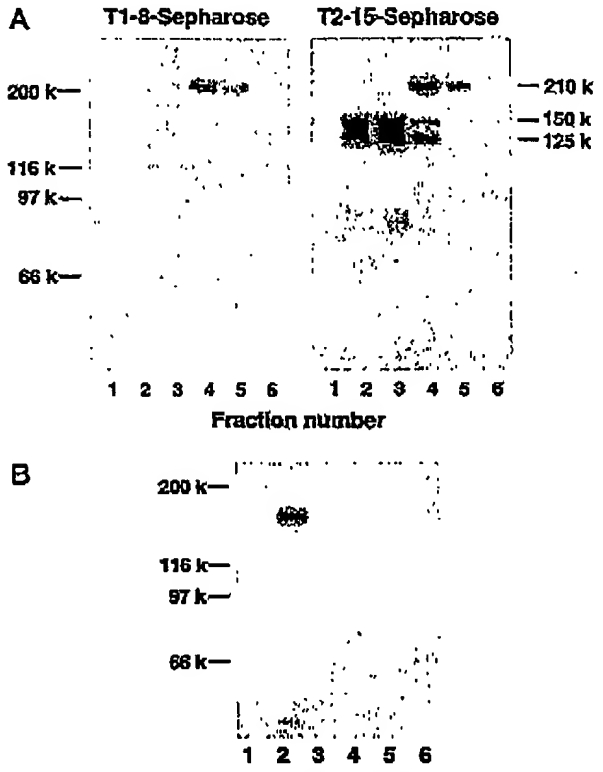

FIG. 8. **T2-15 peptide binds α4β1 integrin complex from B16 cells.** *A*, affinity chromatography of surface iodinated, detergent-extracted B16 cells on T2–15- and T1-8-Sepharose. Fractions 1–6 represents material eluted with 5 mM EDTA and samples were subjected to SDS-PAGE under reducing conditions on a 7.5% polyacrylamide gel. Note that polypeptides of relative molecular masses of 150 and 125 kDa bound only to T2–15-Sepharose. The positions of molecular mass standards are indicated on the *left*. *B*, immunological analysis of T2–15-Sepharose binding material with anti-integrin antibodies. The EDTA-eluted fractions from T2–15-Sepharose were immunoprecipitated with the following antibodies, and analyzed on SDS-PAGE on 7.5% gel. *Lane 1*, polyclonal antiserum recognizing α3B; *lane 2*, polyclonal antiserum recognizing α4; *lane 3*, polyclonal antiserum recognizing α5; *lane 4*, monoclonal antibody GoH3 IgG recognizing α6β1; *lane 5*, polyclonal antiserum recognizing α7B; and *lane 6*, polyclonal antiserum recognizing αv. The final concentrations were 2 μg/ml for pure IgG, and 1:200 for antisera.

VLA-4 integrin, its reactivity toward available immunological probes for the various integrin α subunits was examined. The EDTA-eluted proteins were subjected to immunoprecipitation using antisera against the cytoplasmic tail of integrin subunits α3, α4, α5, α7, αv, and an anti-human α6 rat mAb which can also recognize mouse α6 (GoH3). As clearly shown in Fig. 8*B*, only anti-α4 antibody could immunoprecipitate radiolabeled polypeptide from the material eluted from T2–15-Sepharose. The relative molecular mass of the polypeptide is about 150 kDa, suggesting that the 125-kDa band (β1 subunit) was lost during the immunoprecipitation procedure. This is consistent with a notion that α4β1 integrin complex tends to be dissociated during immunoprecipitation experiments conducted in the presence of EDTA (14, 31). It is concluded that T2–15 peptide can directly bind to VLA-4 complex in the presence of divalent cation and contains a novel ligand sequence for the VLA-4 integrin.

DISCUSSION

We report herein that the receptor responsible for cell adhesion to pp-vWF is VLA-4 (α4β1 integrin). At first we tested more than 20 cell lines, which grow on culture dishes, for their ability to adhere to pp-vWF and found that only the cultured tumor cell lines with melanoma origin, including mouse melanoma B16, human melanoma G-361 and MeWo, and hamster melanoma RPMI 1846, adhered on pp-vWF substrate (data not shown). We then thought that the receptor for pp-vWF might be a melanoma-specific molecule. When we tested cells of hematopoietic origin, however, most of those floating cells attached well to pp-vWF. It became clear that the receptor responsible for adhesion to pp-vWF was rather widely distributed among hematopoietic cells but its expression on adherent cells was limited to several melanoma cells. In a previous paper (8), we reported that the cell adhesion to pp-vWF was mediated by the β1 class of integrin using a function-blocking anti-β1 mAb, but could not identify the corresponding α subunit. Today 10 α subunits (α1–9 and αv) are known to be associated with β1 integrin. Among those, α4β1 integrin (VLA-4) is known as a rather common antigen on hematopoietic cells (32) and it is also known that several melanoma cells express high levels of VLA-4 (33). As the expression of α4 but not other α subunits correlated with the adhesive activity of hematopoietic cells to pp-vWF, we tested the effect of anti-α4 mAbs on the cell adhesion to pp-vWF and found they completely inhibited adhesion. This strongly indicates that VLA-4 is the receptor for pp-vWF. Other mAbs with function blocking ability, including anti-α2(6F1), α3(P1B5), α5(BIIG2), α6(GoH3), and αv(LM609), did not affect adhesion (data not shown), ruling out these subunits as receptor candidates. Finally, transfection of α4 integrin cDNA into CHO cells resulted in acquisition of adhesion activity toward pp-vWF as well as other well known ligands; CS1 and VCAM-1. It is concluded at this point that the cell adhesion receptor to pp-vWF is α4β1 integrin.

VLA-4 is an integrin complex that recognizes both CS1 region in fibronectin and VCAM-1. Lymphocyte adhesion to endothelial cells is primarily mediated by interaction of lymphocyte VLA-4 with VCAM-1 expressed on cytokine-activated endothelial cells and this pathway is thought to be central to the lymphocyte recruitment to the site of inflammation (34, 35). In addition, VLA-4/VCAM-1 interaction is involved in lymphocyte homing to high endothelial venule in peripheral lymph nodes where VCAM-1 is constitutively expressed (36). Leukocyte extravasation at the site of inflammation as well as the vascular wall injury is also thought to be mediated by leukocyte integrins, including VLA-4 although other adhesion receptors such as selectins play some part (10). These adhesions need to occur immediately at the right place, which means the adhesive ligands must be distributed spatially and timely. Fibronectin is a molecule abundantly found in almost all tissues and its distribution is hard to be controlled by immediate inflammatory response. VCAM-1 is an inducible cell surface molecule on endothelial cells and is a desirable docking molecule for leukocyte recruitment. However, VCAM-1 must be newly synthesized before expression on the cells and it is only detected after 2 h treatment of cultured endothelial cells with tumor necrosis factor-α (37). This means that VCAM-1 cannot mediate leukocyte adhesion in the very early steps in the inflammation. VCAM-1/VLA-4 pathway also cannot work when the endothelial cells are seriously injured or removed from the vascular wall. If there is another molecule that can mediate leukocyte adhesion in the very early step of inflammatory response, the whole system would be more effective. Vonderheide and Springer (38) have suggested that there is an unknown ligand for VLA-4 on endothelium because VLA-4-dependent adhesion of lymphoid cells to cultured endothelial cells is not completely inhibited by antibodies against VCAM-1 and fibronectin. Hahne *et al.* (39) have also suggested that there is an unknown

leukocyte adhesion mechanism on mouse endothelioma cells that is not mediated by VCAM-1, ICAM-1, E-selectin, or P-selectin. Our results that VLA-4 also recognizes pp-vWF as an adhesion substrate may suggest that the adhesion to pp-vWF functions as a physiologically important pathway of leukocyte recruitment to the inflammatory/vascular injury sites. Biosynthesis of vWF precursor is limited in megakaryocytes and endothelial cells (4). Only platelets and endothelial cells are places that pp-vWF protein exists in the whole body and pp-vWF is immediately released from these cells upon activation by agonists such as thrombin (40). As pp-vWF has the ability to bind to both collagen and laminin (5, 7), it may be deposited at the exposed subendothelial matrix, thus presenting an adhesion site for VLA-4-bearing leukocytes. In preliminary experiments, we have observed that pp-vWF rapidly accumulates at injured sites of glomerular vasculature during the experimental glomerulonephritis while mature vWF does not.[2] It is possible that pp-vWF functions as an emergency tag for leukocytes/lymphocytes targeting and mediates successful recruitment and infiltration of these inflammatory cells together with VCAM-1.

We found that an amino acid sequence, DCQDHSFSIVI-ETVQ, derived from the midregion of human pp-vWF, was a novel ligand sequence of VLA-4. A peptide with this sequence by itself could promote cell adhesion in an α4β1 integrin-dependent manner, and, moreover, direct binding of the VLA-4 integrin complex to this peptide is observed. Although the shorter version of the peptide, T2–10 (DCQDHSFSIV), also had cell adhesion activity, the inhibitory activity of this peptide on cell adhesion to pp-vWF was significantly lower than that of T2–15. It suggests that the 10-residue portion is essential for the recognition by VLA-4, but the C-terminal extension increases the affinity and/or stabilizes the favorable conformation of the peptide. CS1 and T2–15 peptide both can inhibit the cell adhesion to pp-vWF as well as to CS1-bovine serum albumin, although these peptides share no homologous motif in their sequences. It is possible that both peptides can bind to the ligand binding pocket of VLA-4 in a similar way, thus inhibiting the interaction of each other. Although the tertiary structure for the pp-vWF protein has not been resolved, a computer-aided prediction of secondary structure of the region containing this sequence by using the algorithm of Robson (41) suggests that the segment Asp^{396}-Phe^{401} form a β-turn structure (data not shown). It is possible that this segment forms loop structures facing outward and is thus available to interact with the cell surface. As this sequence contains neither the essential VLA-4-recognition motif identified in CS1 (EILDV) (42) nor that in VCAM-1 (QIDSPL) (43), it seems that this sequence represents a novel integrin-binding motif. However, it contains a sequence, VIET, which is similar to the sequence around the essential glutamate residue (Glu^{34}) in ICAM-1 (GIET) (44). As these sequences all contain some oxygenated residues (Asp, Glu, Thr, or Ser) as well as isoleucine residue, these different but rather similar sequences might be utilized as general integrin binding motifs in each molecule together with other structures that define the specificity. Determination of minimum essential residues for the binding to the integrin within the T2–15 sequence is underway.

In conclusion, our finding that pp-vWF serves as a ligand for VLA-4 integrin provides new insights on the physiological significance of this vWF gene product which was originally thought as a mere propeptide without any particular functions. Furthermore, elucidation of mechanisms of the cell adhesion to pp-vWF will help in our understanding of mechanisms underlying the melanoma metastasis as well as vascular inflammation.

Acknowledgments—We thank Drs. C. Morimoto, M. E. Hemler, B. S. Coller, C. Damsky, A. Sonnenberg, E. Ruoslahti, D. Dottavio, and E. Wayner for their valuable gifts.

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0022-1767/93/1511-149$02.00/0
The Journal of Immunology

Vol 151, 149-158, No. 1, July 1, 1993
Printed in U.S.A.

Activated T-Cell Adhesion to Thrombospondin Is Mediated by the α4β1 (VLA-4) and α5β1 (VLA-5) Integrins[1]

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ABSTRACT. T lymphocytes utilize adhesion receptors in a regulated manner to interact with other cells and with components of the extracellular matrix. These cell-cell and cell-matrix interactions serve a critical role in T cell recognition of foreign Ag and in the migration of T cells to various anatomic sites in vivo. Thrombospondin is an extracellular matrix protein that is transiently expressed at high concentration in damaged and inflamed tissue. Given recent evidence implicating a role for the extracellular matrix in modulating T-cell migration and function, we analyzed T-lymphocyte interactions with thrombospondin. We show here that $CD4^+$ T cells specifically adhere to thrombospondin predominantly via the 70 kDa core region of the thrombospondin molecule. Antibody blocking and affinity chromatography analysis suggest that T-cell adhesion to thrombospondin involves three distinct receptors: an activation-independent receptor that mediates adhesion of resting T cells, and the α4β1 and α5β1 integrins, which mediate a rapid increase in adhesion to thrombospondin upon activation. These three molecules appear to be novel thrombospondin receptors, as other receptors previously implicated in the adhesion of non-lymphoid cells to thrombospondin appear not to be involved in T-cell/thrombospondin interactions. The up-regulation of α4β1 and α5β1 functional activity upon activation is associated with the preferential adhesion of memory T cells to thrombospondin. Our results thus define three novel thrombospondin receptors, and provide additional evidence that extracellular matrix proteins play an important role in lymphocyte migration into, and retention in, inflammatory sites. *Journal of Immunology*, 1993, 151: 149.

The process of immune surveillance requires the systemic and continuous distribution of the total pool of potentially reactive T lymphocytes through the various anatomic sites in the body. Consequently, molecules that mediate the physical interaction of T cells with the extracellular environment serve an absolutely essential role in T-cell function (1). These adhesion molecules are critical not only to the cell-cell interactions that are required for efficient T-cell-mediated recognition of foreign Ag (2), but also for the specific dissemination of T cells to various anatomic sites (3). The functional activity of T-cell adhesion molecules must also be exquisitely regulated, because T-cell function depends on the appropriate switching between adhesive and nonadhesive states.

The members of the integrin family of adhesion molecules serve to mediate the adhesion of many different cell types, including T cells, to other cells and to components of the extracellular matrix (ECM)[3] (4). There are at present over 20 different integrin receptors, each consisting of an α chain noncovalently associated with a β chain on the cell

Received for publication December 1, 1992. Accepted for publication March 26, 1993.

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[1] Supported by NIH Grants AI-3112601 (YS), CA-51088 (RY and VD) and American Cancer Society Junior Faculty Research Award JFRA-331 (YS).

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[3] Abbreviations used in this paper: ECM, extracellular matrix; FN, fibronectin; LN, laminin; TSP, thrombospondin.

surface (1, 5–7). Human CD4+ T cells express the β2 integrin LFA-1 as well as four integrins of the β1 or VLA subfamily: α3β1 (also designated VLA-3), α4β1 (VLA-4), α5β1 (VLA-5), and α6β1 (VLA-6) (8–10). As seen with other cell types that express these integrins, T cells can interact via these receptors with cell surface molecules such as the LFA-1 ligands ICAM-1, ICAM-2, and ICAM-3, the α4β1 ligands VCAM-1 and invasin, as well as ECM proteins such as FN and LN. Analysis of T-cell integrin function has also revealed that T-cell adhesiveness can be rapidly modulated by T-cell activation events. Resting CD4+ T cells express β1 and β2 integrins but they do not bind strongly to human ligands such as ICAM-1, VCAM-1, FN, and LN. However, activation of T cells with various stimuli, such as the phorbol ester PMA or CD3/TCR cross-linking, results in strong adhesion to these ligands without a change in the level of T-cell integrin expression (10–13).

The identification of functional β1 integrins on human T cells (10, 14) focused attention on the importance of the ECM in regulating T-cell adhesion and migration. Activation-dependent adhesion of CD4+ T cells via β1 integrins to ECM ligands such as FN and LN is likely to play a role in the arrest of lymphocyte migration upon activation (10, 15). Furthermore, treatment of T cells with FN peptides that block α5β1 binding has been shown to prevent T-cell migration to sites of contact hypersensitivity (16); and FN has been implicated in T-cell adhesion to specialized endothelial cells critical to T-cell migration into lymph nodes (17–19). In addition, the differential expression of β1 integrins (and other adhesion molecules) on T-cell subsets functionally defined as naive and memory T cells suggests that integrin-mediated adhesion to the ECM may play a vital role in the differential migration of naive and memory T cells in vivo (3, 20–22). The ability of ECM ligands to facilitate CD3-mediated T-cell proliferative responses suggests that ECM proteins may also serve to modulate T-cell responses to foreign Ag (23–28).

TSP is a 450 kDa protein that is composed of three identical disulfide-linked polypeptides (29–31). TSP is a major component of platelet α-granules and is released upon activation. Endothelial and other cell types also synthesize and secrete TSP, which is subsequently incorporated into the surrounding ECM. In particular, TSP is expressed at high concentrations in damaged and inflamed tissue (32, 33). Analysis of various normal nonlymphoid cell types as well as tumor cells has shown that TSP can have a variety of biologic effects (31, 32, 34) and thus is likely to play a role in normal development as well as in wound repair, tumor metastasis, and inflammatory responses. There is evidence to suggest that cell adhesion to TSP is mediated by multiple distinct receptors, including heparan sulfate proteoglycans (35–40), CD36 (40–43), and the α2β1 (44), αvβ3 (40, 45–47) and gpIIb-IIIa integrins (44).

Given the emerging relevance of the ECM to our understanding of T-cell migration and recognition, and the presence of TSP in the ECM at damaged and inflamed tissue sites, we investigated the interactions of CD4+ T cells with purified TSP. These studies show that T cells adhere to TSP and utilize three receptors that have not been previously identified as TSP receptors on other cell types. Two of these receptors are the α4β1 and α5β1 integrins, which function only after T-cell activation. Furthermore, the preferential adhesion of memory T cells to TSP suggests that TSP may also play a role in the preferential localization of memory T cells at sites of inflammation.

Materials and Methods

Isolation of human peripheral CD4+ T cells

Purified CD4+ T cells were isolated by exhaustive negative magnetic immunoselection from peripheral blood mononuclear leukocytes as previously described (10, 48). Briefly, PBMC were isolated from either leukapheresis packs or buffy coats by Ficoll-Hypaque density-gradient centrifugation. Resting CD4+ T cells were subsequently isolated from PBMC using Advanced Magnetics Particles (Advanced Magnetics, Inc., Cambridge, MA), Dynabeads (Dynal, Inc., Great Neck, NY) and a cocktail of mAb consisting of: IVA12 (anti-HLA class II), FMC63 (anti-CD19), 3G8 (anti-CD16), NIH11b-1 (anti-CD11b), 63D3 (anti-CD14), B9.8.4 (anti-CD8), and 10F7 (anti-glycophorin). The T-cell populations were typically >96%CD3+CD4+ as assessed by flow cytometric analysis (48). In addition to phenotypic analysis of the T-cell preparations to assure purity, complete depletion of monocytes in the T-cell preparation was determined by the lack of proliferative response to optimal concentrations (1/200 dilution) of PHA (M form) (GIBCO BRL, Grand Island, NY).

Thrombospondin

TSP was purified as previously described (49). Briefly, TSP was isolated from thrombin-stimulated platelet releasate by gelatin and heparin affinity followed by gel filtration chromatography. Purified TSP was concentrated by ultrafiltration and stored at −80°C. Lack of FN in the TSP preparations was confirmed by immunoblotting analysis with anti-FN antibodies (not shown).

mAb and other reagents

The following mAb specific for the indicated T-cell surface Ag were used at a concentration of 10 μg/ml: anti-CD43 mAb 84–3C1, anti-CD45 mAb NIH45–2 (50), anti-CD59 mAb BRIC229 (D. Anstee, Bristol), anti-CD38 mAb OKT10 (ATCC, Rockville, MD), anti-CD7 mAb 3A1 (ATCC), anti-E2 mAb TU12 (A. Ziegler, Tubingen), anti-CD28 mAb 9.3 (J. Ledbetter, Seattle), anti-CD36 mAb OKM5 (Ortho, Raritan, NJ), anti-CD44 mAb NIH44–1

(51), anti-CD26 mAb 5/9, anti-β1 mAb 4B4 (Coulter Corp., Hialeah, FL), AIIB2 (C. Damsky, San Francisco), and MAB13 (25) (K. Yamada, Bethesda), anti-β3 mAb LM609 (D. Cheresh, LaJolla), anti-β2 mAb MHM23, anti-α3 mAb P1B5 (Telios Pharmaceuticals, Inc., San Diego, CA), anti-α4 mAb L25 (Becton Dickinson, San Jose, CA), anti-α5 mAb MAB16 (25) (K. Yamada, Bethesda). The anti-TSP mAb are specific for the following regions of the TSP molecule (52): 70 kDa core region (A4.1), distal carboxyl terminus (C6.7), heparin binding domain (A2.5), Ca^{++}-binding region (D4.6), 70 kDa core region (A6.1). All of the anti-TSP mAb were used as purified mAb at a concentration of 20 μg/ml. EDTA was used at a final concentration of 5 mM and heparin was used at a concentration of 50 μg/ml. Stock solutions of PMA (Sigma Chemical Co., St. Louis MO) were dissolved in DMSO.

Adhesion assays

Adhesion assays were performed as previously described (10). Briefly, purified TSP was applied to 96-well flat bottom plates (Costar Corp., Cambridge, MA) at the concentrations indicated in PBS (always containing Ca^{2+}/Mg^{2+}) overnight at 4°C. Unbound sites were subsequently blocked with PBS/2.5% BSA for 2 to 3 h at 37°C and 50,000 ^{51}Cr-labeled T cells were added to each well in a final volume of 100 μl of PBS/0.5% human serum albumin (HSA). For PMA activation, cells were added to wells containing 10 ng/ml PMA. Blocking mAb were added to appropriate wells and were present throughout the assay. After 1 h settling at 4°C, plates were rapidly warmed to 37°C for 10 min, nonadherent cells washed off, and the percentage of bound cells determined by lysing with detergent, and counting gamma emissions from the well contents. Data are expressed as the mean percent of cells binding from triplicate wells.

Panning assays were performed by incubating purified $CD4^+$ T cells (10×10^6 cells/well) in 4-well tissue culture plates (Linbro, Irvine, CA) coated as described with TSP (50 μg/ml) either in the absence or presence of 10 ng/ml PMA for 1 h at 4°C and then rapidly warming the plates to 37°C for 10 min. Nonadherent cells were removed by three washes with PBS containing Ca^{2+} and Mg^{2+}; these cells represent the TSP-nonadherent cells. Cells remaining after the washes were removed by incubation in 1 mM EDTA and collected; these cells represent TSP-adherent cells. All fractions, including an aliquot of the original $CD4^+$ T-cell population, were subsequently analyzed for expression of either CD45RO using the anti-CD45RO mAb UCHL1 (Dako Corp., Carpinteria, CA) or CD45RA using the anti-CD45RA mAb IOL2 (Amac, Inc., Westbrook, ME) by flow cytometry (8, 10).

Flow cytometry

Single-color flow cytometric analysis was performed essentially as described (8, 10). Cells were stained by incubating with saturating amounts of PE-conjugated CD45RO-specific mAb UCHL1 or FITC-conjugated CD45RA-specific mAb IOL2 and subsequently analyzed on a FACScan (Becton Dickinson, San Jose, CA).

Affinity chromatography

Resting or PMA-activated $CD4^+$ T cells ($50–200 \times 10^6$ total cells) were iodinated in PBS with 0.4 to 2.0 mCi $Na^{125}I$ by the Iodogen method for 30 to 60 min on ice. Cells were washed four times to eliminate free counts and resuspended in 200 to 400 μl of A buffer (20 mM HEPES, pH 7.2, 0.15 M NaCl, 1 mM Ca^{2+}, 1 mM Mg^{2+}, 0.3% CHAPS containing protease inhibitors (1 μg/ml each of pepstatin, leupeptin, aprotinin and 10 μg/ml of soybean trypsin inhibitor)). Detergent extraction was carried out for 1 h on ice with occasional vortexing after which the soluble fraction was recovered by centrifugation at 14,000*g* for 30 min at 4°C. TSP affinity chromatography was performed as described (53) with the following modifications: iodinated extracts were batch incubated with TSP affinity beads overnight at 4°C before pouring into a column and washing extensively. Bound material was eluted sequentially in A buffer containing 5 mM EDTA/EGTA followed by 1 M NaCl, fractions pooled, and TCA precipitated or subjected to immunoprecipitation. For resting T cells 5 mM EDTA/EGTA specifically eluted virtually all bound material, and no counts were further eluted with 1 M NaCl. The anti-β1 mAb LM534 (D. Cheresh, LaJolla) was used at a 1:20 dilution to immunoprecipitate material eluted from the TSP affinity column. Immunoprecipitated material was processed as described (53). All samples were incubated in nonreducing gel sample buffer and subjected to SDS-PAGE on either 5% (resting) or 7.5% (PMA-activated) gels before being processed for autoradiography.

Results

Adhesion of $CD4^+$ T cells to TSP

Resting $CD4^+$ T cells adhere in a dose-dependent fashion to purified TSP immobilized on plastic, with maximal adhesion at 2.5 μg TSP/well (Fig. 1). Furthermore, activation of T cells with PMA resulted in increased adhesion to TSP at all doses of TSP tested. The specificity of adhesion was shown using mAb specific for different functional regions of TSP (52). mAb A4.1 specific for the 70 kDa core region of TSP almost completely inhibited adhesion of both resting and PMA activated T cells (Fig. 2). These results are consistent with other studies demonstrating a role for the TSP core region in melanoma and endothelial cell adhesion (54, 55). mAb C6.7 specific for the distal carboxyl terminus of

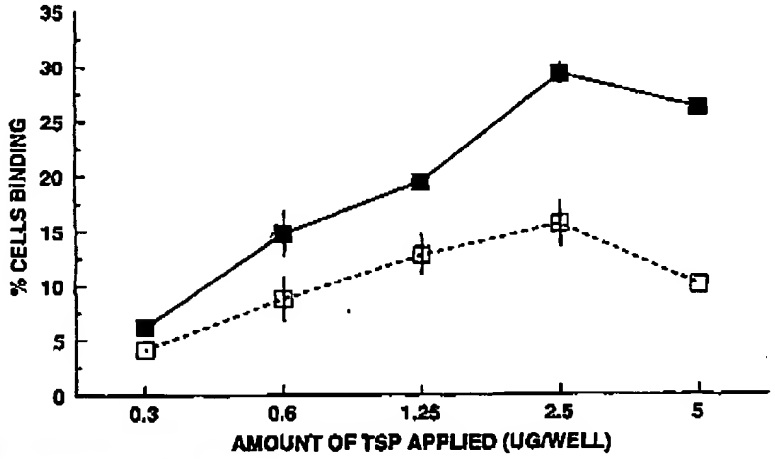

FIGURE 1. Adhesion of resting and PMA-activated CD4+ T cells to TSP. Adhesion of ^{51}Cr-labeled CD4+ T cells to purified TSP before (*open squares*) or after activation for 10 min at 37°C with 10 ng/ml PMA (*solid squares*) was assessed as described in *Materials and Methods*. Binding of resting and PMA-activated T cells to the negative control protein collagen was <7% and has not been subtracted from the values shown. Results are representative of a minimum of five independent experiments with T cells isolated from different donors and different preparations of TSP.

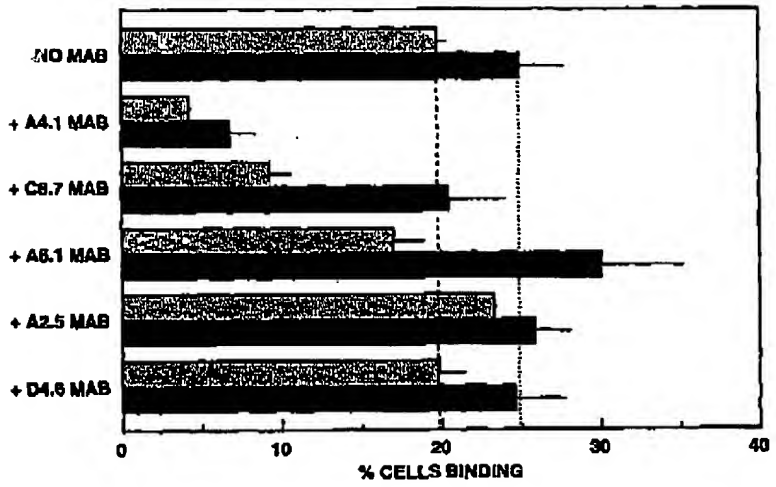

FIGURE 2. Blocking of CD4+ T-cell adhesion to TSP by anti-TSP mAb. Adhesion of resting (*shaded bars*) and PMA-activated (*solid bars*) CD4+ T-cell adhesion to TSP (2.5 μg/well) was assessed in the presence of the indicated mAb specific for different functional regions of TSP. All mAb were present throughout the assay at 20 μg/ml. Binding of resting and PMA-activated T cells to the negative control protein collagen was <4% and has not been subtracted from the values shown. Results are representative of a minimum of five independent experiments.

TSP partially inhibited adhesion of resting T cells to TSP, but had only a minimal inhibitory effect on adhesion of PMA-activated T cells. None of the other TSP-specific mAb tested, including mAb A2.5 specific for the heparin-binding domain, inhibited adhesion of resting or activated T cells.

Adhesion of resting CD4+ T cells to TSP is mediated by an undefined receptor

To identify potential TSP receptors, mAb against various T-cell surface molecules were tested for inhibition of adhesion. None of the mAb tested inhibited adhesion of resting T cells to TSP (Fig. 3). Two cell surface molecules implicated in the adhesion of other cell types to TSP, CD36 and the αvβ3 integrin (40–43, 45–47), are not expressed on human T cells (data not shown), and mAb against these molecules did not affect T-cell adhesion to TSP (Figs. 3 and 4). The addition of sodium heparin also failed to inhibit the adhesion of both resting and PMA-activated T cells to TSP (Fig. 5), suggesting that heparan sulfate proteoglycans were not involved in T-cell adhesion to TSP (35–40). However, the ability of EDTA to completely inhibit T-cell adhesion to TSP suggested that the function of these novel TSP receptors is dependent on divalent cations (Fig. 5).

Inhibition of PMA-activated CD4+ T-cell adhesion to TSP by anti-β1 mAb

The ability of PMA activation to increase T-cell adhesion to TSP was consistent with the involvement of integrins, as previous studies have shown that T-cell activation results in the rapid up-regulation of integrin functional activity (10–12). Although functionally inhibitory mAb against β1 and β2 integrins failed to inhibit resting T-cell adhesion to TSP (Figs. 3 and 4), mAb specific for the β1 chain, but not the β2 chain, were able to inhibit adhesion of PMA-activated T cells to TSP down to the levels of adhesion seen with resting T cells (Fig. 4). These results suggest the involvement of β1 integrins in the adhesion of PMA-activated T cells to TSP.

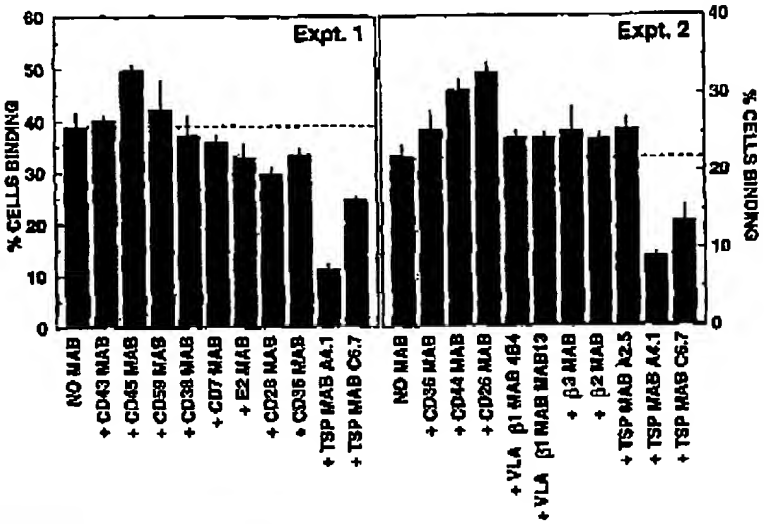

FIGURE 3. Lack of inhibition of resting T-cell adhesion to TSP by various mAb. Adhesion of resting CD4+ T cells to TSP in the presence of the indicated mAb specific for various T-cell surface molecules was assessed. All mAb were used at a concentration of 10 μg/ml, except for the anti-TSP mAb A4.1 and C6.7, which were used at 20 μg/ml. TSP was applied at 1 μg/well in both experiments shown.

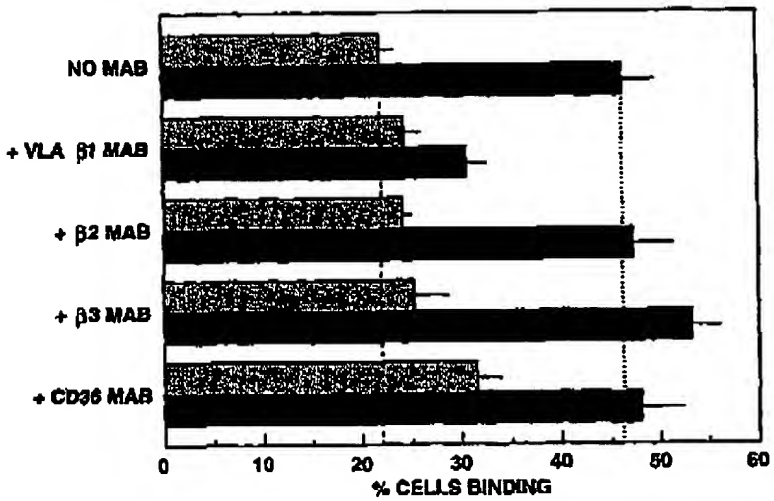

FIGURE 4. Anti-β1 mAb inhibit the adhesion of PMA-activated T cells but not resting T cells to TSP. Blocking of adhesion of PMA-activated T cells (*solid bars*) but not resting T cells (*shaded bars*) to TSP in the presence of the following mAb: anti-β1 mAb 4B4, anti-β2 mAb MHM23, anti-β3 mAb LM609, and anti-CD36 mAb OKM5. All mAb were used at a concentration of 10 μg/ml. Similar inhibition of PMA-activated T-cell adhesion to TSP was also observed with the anti-β1 mAb MAB13 (not shown). Adhesion to the negative control protein collagen was <6% and was not subtracted from the values shown. TSP was applied at 1 μg/well.

Adhesion of PMA-activated CD4+ T cells can be inhibited by mAb specific for the integrin α4 and α5 chains

Previous studies have shown that human CD4+ T cells express the α4β1, α5β1 and α6β1 integrins, as well as low but detectable levels of the α3β1 integrin (10); resting CD4+ T cells do not express either α1β1 or α2β1. Functionally inhibitory mAb specific for individual integrin α chains were used to identify the specific β1 integrins involved in PMA-activated T-cell adhesion to TSP. Individually, mAb specific for the α4 chain or α5 chain partially inhibited adhesion of PMA-activated T cells to TSP; the combination of α4- and α5-specific mAb blocked adhesion as efficiently as the β1-specific mAb, suggesting that α4β1 and α5β1 both mediate PMA-activated T-cell adhesion to TSP (Fig. 6). mAb specific for other α chains, including α3 and α6, failed to inhibit T-cell adhesion to TSP (Fig. 6 and data not shown).

Both α4β1 and α5β1 have also been shown to bind to distinct sites on FN (10, 14). Several lines of evidence clearly demonstrated that T-cell adhesion to TSP via α4β1 and α5β1 is distinct from adhesion to FN. First, a direct comparison of PMA-activated T-cell adhesion to TSP and FN showed differential efficiency of inhibition by the α4- and α5-specific mAb (Fig. 6). Although adhesion to FN is mediated predominantly by α5β1 (Fig. 6) (10), equivalent contributions of α4β1 and α5β1 were seen in adhesion to TSP. Second, some α5-specific mAb that efficiently inhibit T-cell adhesion to FN failed to inhibit adhesion to TSP (data not shown). Furthermore, TSP-specific mAb failed to inhibit T-cell binding to FN and no FN was detectable in our preparations of TSP by Western blotting analysis with FN-specific antibodies (data not shown).

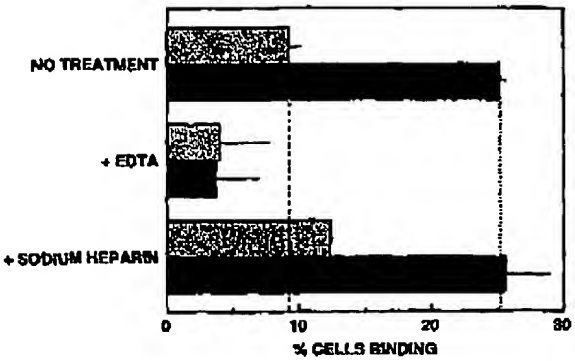

FIGURE 5. CD4+ T-cell adhesion to TSP is EDTA-sensitive and heparin-insensitive. Adhesion of resting (*shaded bars*) and PMA-activated T-cells (*solid bars*) was assessed in the continuous presence of 5 mM EDTA or 50 μg/ml sodium heparin. TSP was applied at 2.5 μg/well. Adhesion to the negative control protein collagen was <6% and was not subtracted from the values shown.

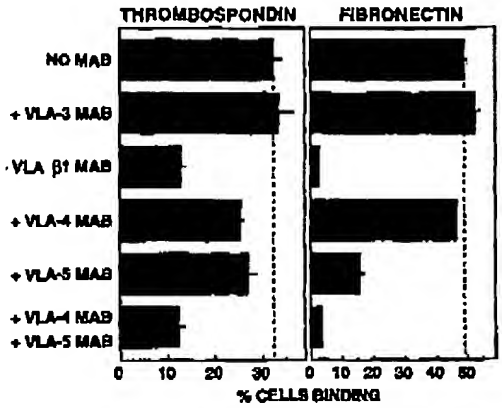

FIGURE 6. Adhesion of PMA-activated CD4+ T cells to TSP involves the α4β1 and α5β1 integrins. Adhesion of PMA-activated T cells to TSP (*left panel*) or FN (*right panel*) in the presence of the following mAb (used at 10 μg/ml): anti-α3 mAb P1B5, anti-β1 mAb AIIB2, anti-α4 mAb L25, and anti-α5 mAb MAB16. TSP was immobilized at 2.5 μg/well and FN at 1 μg/well.

Differential adhesion of PMA-activated CD4+CD45RA+ "naive" and CD4+CD45RO+ "memory" T cells to TSP

β1 integrins and other adhesion molecules are differentially expressed on reciprocal subsets of CD4+ T cells functionally defined as "naive" and "memory" T cells (10, 56, 57). Isoforms of the CD45 tyrosine phosphatase are the best discriminators of naive and memory T cells, with greater expression of the CD45RA isoform on naive T cells and greater expression of the CD45RO isoform on memory T cells. The greater expression of β1 integrins on CD4+CD45RO+ memory T cells compared to CD4+CD45RA+ naive T cells is associated with greater memory T-cell adhesion to FN, VCAM-1, and LN (10, 58). Panning of CD4+ T cells on TSP-coated plastic surfaces and subsequent flow cytometric analysis of the TSP-adherent and nonadherent populations using CD45RO- and CD45RA-specific mAb also demonstrated preferential adhesion of PMA-activated CD4+CD45RO+ memory T cells to TSP (Fig. 7). Interestingly, resting naive and memory CD4+ T cells bound equally well to TSP, suggesting that the TSP receptor mediating resting T-cell adhesion to TSP may be equally expressed on these two T-cell subsets.

Identification of β1 integrins as T-cell TSP receptors by affinity chromatography

Our in vitro adhesion studies implicated at least three TSP receptors on T cells, one undefined receptor active on resting T cells, and the α4β1 and α5β1 integrins, which mediate TSP adhesion after T-cell activation. To confirm these findings, lysates of radioiodinated resting or PMA-stimulated T cells were chromatographed over a TSP affinity matrix, and eluted with either EDTA/EGTA or high salt (Fig. 8). Bands of 80 and 90 kDa were present in the EDTA/EGTA eluates from both resting and PMA-stimulated cells. In addition, resting T cells showed an additional 120 kDa band and PMA-stimulated T cells had an additional 100 kDa band. The high salt eluate from PMA-stimulated T cells shared the 80, 100, and 120 kDa bands with the EDTA/EGTA eluate, but also contained a 75 kDa band. Longer autoradiographic exposures of these eluates showed the presence of additional bands at molecular weights characteristic of the α4 and α5 integrin chains (not shown). Immunoprecipitation of the eluate with β1-specific mAb showed three bands of 120, 140 and 160 kDa (Fig. 8 and data not shown), consistent with the reported molecular mass of the β1, α4 and α5 chains respectively (5). Additionally, the 75 and 80 kDa bands were identified as proteolytic fragments of the α4 chain by immunoprecipitation with α4-specific mAb (data not shown). The unidentified bands at 90 and 100 kDa might represent candidates for the TSP receptor active on resting T cells.

Discussion

These studies demonstrated that human CD4+ T cells adhere to the ECM protein TSP via three distinct receptors. One is a presently undefined receptor that is active on resting T cells and unaffected by T-cell activation. The other two receptors are the α4β1 and α5β1 integrins, which are functionally active only after T-cell activation. All three of these molecules have not been previously implicated in cell adhesion to TSP. In particular, neither α4β1 or α5β1 have been previously shown to mediate adhesion to TSP, and these studies are the first to demonstrate that α5β1 can bind to a ligand other than FN.

Previous studies of nonlymphoid cells have implicated

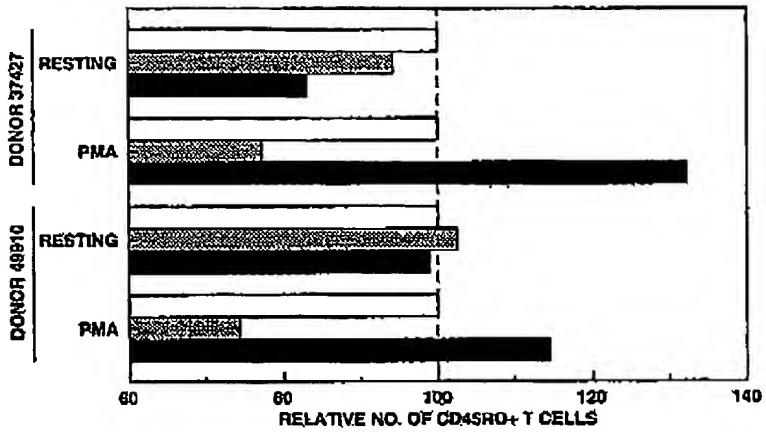

FIGURE 7. Greater β1 integrin expression on CD4⁺CD45RO⁺ "memory" T cells is associated with greater adhesion of PMA-activated CD4⁺CD45RO⁺ T cells to TSP. Preferential adhesion of CD4⁺CD45RO⁺ memory T cells to TSP was assessed by panning as described in *Materials and Methods*. The resulting TSP-adherent and nonadherent cell populations, along with an aliquot of the original population of T cells, were analyzed by flow cytometry for expression of either the CD45RO isoform (Donor 37427) or the CD45RA isoform (Donor 49910). Data are presented as the number of CD45RO⁺ T cells in the TSP-adherent (*solid bars*) and nonadherent (*hatched bars*) fractions relative to the percentage of CD45RO⁺ T cells in the original CD4⁺ T cells (normalized to 100, shown in *open bars* for each donor). The actual percentage of CD45RO⁺ T cells before panning was 56% for Donor 37427 and 40% for Donor 49910.

several other cell surface molecules as TSP receptors. However, none of these molecules appears to mediate the adhesion of CD4⁺ T cells to TSP. Heparan sulfate proteoglycans have been shown to be involved in the adhesion of various cell types to TSP (35–40). However, the inability of sodium heparin or mAb A2.5 specific for the heparin-binding domain of TSP to inhibit the adhesion of either resting or PMA-activated T cells to TSP suggests that heparan sulfate proteoglycans are not involved in T cell-TSP interactions. The CD36 molecule has also been implicated as a TSP receptor (59) on platelets (41, 42), fibroblasts (40) and tumor cells (43). However, the role of CD36 in TSP binding remains controversial, as CD36 expressed in COS monkey kidney cells did not result in adherence to TSP (60) and CD36 mAb do not inhibit adhesion of the CD36⁺ cell line U937 to TSP (61). Integrins that have been implicated as potential TSP receptors include α2β1 and gpIIb-IIIa on platelets (44), and αvβ3 on endothelial cells (45, 46), melanoma cells (46), embryonic fibroblasts (40), and platelets (47). However, CD36, α2β1, αvβ3, and gpIIb-IIIa are not expressed on peripheral CD4⁺ T cells (J0 and data not shown) and consequently mAb against these molecules fail to inhibit T-cell adhesion to TSP. Human neutrophils also adhere to TSP (62), but T-cell adhesion to TSP appears to be distinct from that of neutrophils, because the anti-TSP mAb A6.1 inhibited neutrophil but not T-cell adhesion to TSP (62). Thus, our studies provide important new evidence that other integrins, such as α4β1 and α5β1, can mediate the adhesion of human lymphoid cells to TSP.

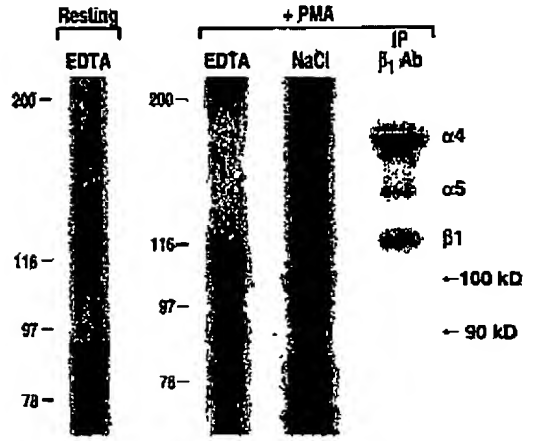

FIGURE 8. TSP affinity chromatography of radioiodinated resting and PMA-activated CD4⁺ T cells demonstrates α4β1 and α5β1 mediation of T-cell binding to TSP. Iodinated resting or PMA-activated T-cell extracts were chromatographed over a TSP affinity matrix and eluted with either 5 mM EDTA/EGTA (*EDTA lanes*) or 1 M NaCl (*NaCl lane*). Immunoprecipitation of the eluate was performed with the anti-β1 mAb LM534 (*IP β1 Ab lane*). Molecular weight standards (in kDa) are as indicated.

Previous studies did not uncover a role for either α4β1 or α5β1 in cell adhesion to TSP, even though many of the cell types studied express α5β1. It is possible that on cell types that express other TSP receptors, such as heparan sulfate proteoglycans, CD36 and αvβ3, α5β1 may serve as a weak TSP receptor that is not detectable by antibody blocking studies or affinity chromatography. It is also possible that there are cell-specific differences in the ability of either α4β1 or α5β1 to bind to TSP, because: 1) α2β1 has

been shown to differentially recognize LN depending on the cell type in which it is expressed (63); 2) activated T cells express three β1 integrins (α1β1, α2β1, and α3β1) that have been shown to bind to collagen on other cell types, but only α2β1 plays a role in activated T-cell binding to collagen (64); and 3) T-cell adhesion to the β1 bacterial ligand invasin is mediated only by α4β1, even though α5β1 has been shown to mediate the binding of other cell types to invasin (65). Thus, it is possible that cell-specific differences may be responsible for the lack of previous identification of α4β1 and α5β1 as TSP receptors. Furthermore, the generally restricted distribution of α4β1 to cells of the hematopoietic lineage (5) suggests that a role for α4β1 as a TSP receptor may not have been uncovered in previous studies involving nonlymphoid cells. It is interesting to note that 24 h treatment of the monocytoid cell line U937 with PMA also resulted in increased adhesion to TSP (61), suggesting a potential role for β1 integrins in U937 adhesion to TSP.

The identity of the receptor mediating the adhesion of resting T cells to TSP remains unknown. Various mAb against T-cell surface molecules failed to inhibit resting T-cell adhesion to TSP, although the unidentified 90 and 100 kDa bands that are eluted off the TSP affinity column may represent this receptor. The relationship of this receptor to the 105/80 kDa receptor on squamous carcinoma cells is also not known (53). However, the ability of the anti-TSP mAb A4.1 specific for the 70 kDa core region of TSP to dramatically inhibit resting T-cell adhesion to TSP suggests that these two receptors might be distinct.

Utilization of multiple distinct receptors for adhesion of T cells to TSP is consistent with other studies demonstrating a cooperative interaction between heparan sulfate proteoglycan, CD36, and αvβ3 in the adhesion of fibroblasts to TSP (40). It is also consistent with the ability of both α4β1 and α5β1 to mediate T-cell adhesion to FN (10). Multiple distinct receptors acting together to mediate cell-ECM interactions is a common theme that is likely to be critical in providing various levels by which the strength of these interactions can be regulated. In the case of T cells, the unidentified TSP receptor on resting T cells may serve to weakly tether T cells to TSP in the surrounding matrix; this weak interaction may serve to immobilize T cells in a tissue site long enough for an activation event to occur, which would subsequently increase the adhesion to TSP (and other ECM proteins) by up-regulating the activity of T-cell β1 integrins. Thus, the utilization of multiple receptors to mediate adhesion to a single ECM protein allows for very precise regulation of the adhesive event. The identification of two new, distinct TSP genes (66–68) suggests that additional regulation at the level of the ECM ligand could also differentially modulate T-cell adhesion to TSP.

Our results provide further evidence for the importance of the ECM in T cell-adhesion and demonstrate that T cells can interact with multiple ECM components, including TSP. The expression of TSP at high levels in inflamed and damaged vascular tissue (32, 33) suggests that TSP may be critical to T cell-migration in inflammatory sites. TSP secretion by activated, proliferating endothelial cells or activated platelets may provide a TSP-rich surface with which CD4+ T cells and potentially other lymphocytes can interact. There are several lines of evidence that are consistent with this hypothesis. First, we have shown in this study that PMA-activated CD4+CD45RO+ memory T cells show greater adhesion to TSP than CD4+CD45RA+ naive T cells. These results provide further evidence for the involvement of β1 integrins in PMA-activated T-cell binding to TSP, and suggest that TSP may be involved in the preferential localization of memory T cells to inflammatory sites in vivo (3, 20–22). Second, anti-TSP mAb have been shown to inhibit the adhesion of PMA-activated U937 cells to rat endothelial cells (61). Third, the potential role of cell surface FN in lymphocyte adhesion to high endothelial venules (17–19) further emphasizes the significance of the ECM to cell-cell interactions that are critical to lymphocyte migration. Furthermore, the ability of other ECM components to modulate immune responses in vitro (23–28) suggest that TSP might also affect T-cell responses. Further investigation of T-cell interactions with TSP is likely to continue to yield important new insights into the role of TSP, and the ECM in general, in regulating lymphocyte migration and function.

Acknowledgments

We thank E. Ennis for technical assistance, the University of Michigan Hospitals Blood Bank and the American Red Cross of Southeastern Michigan for assistance in obtaining blood, and the many investigators who provided mAb.

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